

*Third ACTION Scientific Workshop
Transformative Strategies-Development of Pain Therapies*

June 23, 2014

*A Matter of Record
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1 THIRD ACTION SCIENTIFIC WORKSHOP

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4 TRANSFORMATIVE STRATEGIES FOR THE

5 DEVELOPMENT OF PAIN THERAPIES

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11 Monday, June 23, 2014

12 8:30 a.m. to 5:06 p.m.

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19 FDA White Oak Campus

20 White Oak Conference Center

21 Building 31, The Great Room

22 Silver Spring, Maryland

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1 P R O C E E D I N G S

2 (8:30 a.m.)

3 DR. DWORKIN: All right. It's 8:30. I'm

4 Robert Dworkin from the University of Rochester, and

5 I'd like to welcome you all to this ACTION

6 scientific workshop.

7 ACTION, for those of you who don't know,

8 stands for analgesic, anesthetic and addiction

9 clinical trial translations, innovations,

10 opportunities and networks. It's a public-private

11 partnership with the FDA, and this is our third

12 public scientific workshop. And this one, in

13 distinction to the previous two, is focusing on

14 transformative strategies for the development of new

15 pain therapies.

16 The objectives of this next two days are on

17 some of the materials you've, I think, received by

18 mail, to discuss progress, prospects and

19 opportunities for genetic, epigenetic, and

20 pharmacogenomic research on pain, review ongoing

21 studies of stem cells, gene therapy and toxins in the

22 treatment of pain. And then tomorrow afternoon,

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1 examine recent translations of preclinical findings
2 to successful clinical demonstrations of analgesic
3 efficacy.
4 The steering committee for this meeting are
5 those four individuals, and what I tried to do in the
6 font is indicate the contributions made by those four
7 individuals.
8 Dr. Basbaum is objecting to the font size.
9 He would like his font size to be larger than
10 Dr. Porreca's, but I think we will leave it to the
11 two of them to settle that at the coffee break.
12 But as you can see, the important point that
13 the slide makes is that both Dr. Basbaum and
14 Dr. Porreca, who I think many of you know very well,
15 have really done the lion's share of the work for
16 this workshop. And Dennis and I contributed a
17 miniscule amount. Actually, the fonts for our name
18 are probably too large.
19 The other person I'd like to acknowledge is
20 Dr. Allison Lin here at the FDA, and Allison has
21 really been critically important in arranging the
22 kind of hospitality of the FDA for this meeting.

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1 And, of course, we couldn't do any of the ACTION and
2 IMPACT meetings without the help of Valorie and
3 Andrea, who are responsible for all of the logistical
4 arrangements. So thank you, Allison. Thank you,
5 Valorie. Thank you, Andrea.
6 You should all know that the FDA has required
7 that we tape this meeting, and that's occurring. And
8 both a transcript and video recording will be made
9 available on the ACTION website. It probably takes
10 three to four weeks for that to happen, and it will
11 be a kind of verbatim transcript and also a video
12 recording of the meeting. Both will become
13 available.
14 Then most importantly, the agenda for this
15 meeting is really kind of dense and packed with
16 presentations. And we also want to make sure there's
17 enough time for a discussion. So I'd like to beseech
18 the presenters to please, please keep to whatever
19 amount of time is allotted on the program for your
20 talk.
21 Then if you want to learn anything more about
22 ACTION, there's a tremendous amount of information

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1 on the website. We update this every month or two,
2 and the address is ACTION with two Ts, dot org.
3 Then finally, before I turn it over, some
4 housekeeping. Because we were a little early this
5 morning, we decided to make the buses a little bit
6 later tomorrow morning. So the buses from the hotel
7 will leave at 7:30 rather than 7:15. There will
8 still be ample time, of course, for breakfast. There
9 will be buses or a bus back to the hotel at 5:00 this
10 afternoon from here, and there will be breakfast
11 again tomorrow as there was today.
12 We are required to say that the federal
13 government policy prohibits those individuals who
14 work for the government from partaking in the
15 continental breakfast that the rest of you were able
16 to have this morning. I don't know why we're
17 required to say that, but I have discharged my duties
18 to the U.S. government by just saying that bullet.
19 Then finally, of course, please silence your
20 cell phones.
21 So it's a great pleasure to introduce
22 Dr. Allan Basbaum, who is going to be starting off.

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1 He's actually co-chairing the first session of the
2 meeting with Dr. Bill Maixner.
3 Dr. Basbaum, of course, is a professor at the
4 University of California San Francisco.
5 Dr. Maixner's a professor at the University of North
6 Carolina. And they will be co-chairing -- and
7 neither of them need any introduction at all to this
8 audience. And they will be co-chairing our first
9 session this morning.
10 Allan.
11 DR. BASBAUM: Thank you, Bob, and welcome,
12 everyone. We should put this meeting in a little
13 more perspective. There's been so much in the news
14 lately, and you have to be living on another planet
15 if you haven't read it, about the concerns about the
16 translation from preclinical work, whether it's in
17 particularly cancer or pain, into the clinic, whether
18 it's issues of animal models, reproducibility,
19 statistics, power calculations, you name it.
20 It was becoming a rather intense discussion,
21 and several of us got together and said, well, maybe
22 we need to have a slightly different perspective and

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1 really look to the future instead of keep constantly
2 looking to the past. And by that, we ended up with
3 this meeting, which the word "transformative" is
4 somewhat overused. But that's really what we're
5 looking for, is where does the future lie. What are
6 some of the new approaches, techniques that might be
7 relevant, that hopefully will be relevant, to the
8 development of useful pharmacotherapies -- I
9 shouldn't even say pharmacotherapies -- for chronic
10 pain?
11 That's really the rationale for this meeting.
12 It's very fundamental in nature, but hopefully, we'll
13 get into discussions of how this might be translated
14 in the hopefully not too distant future.
15 So I want to thank everyone for coming. We
16 do encourage discussion. That's what makes these
17 meetings special, and hopefully, we can do that. And
18 it's my pleasure to introduce Steve McMahon, who's
19 not only a good friend but a terrific colleague.
20 We both trained with Pat Wall many years ago.
21 I'm a little older than Steve, so I was there a
22 little bit before him. He is now Sherrington

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1 professor -- which is such a wonderful thing to be; I
2 would love to be a Sherrington professor -- of
3 physiology at Kings College. And he's also -- and
4 this is relevant. He's an academic leader of a
5 public/private partnership, which is somewhat
6 analogous to ACTION, called Europain. And in many
7 respects, their objectives are comparable to those to
8 which ACTION adheres.
9 His interests are diverse, whether it's from
10 mechanisms of neurotrophin action. But most
11 recently, his interest turned to epigenetics, and
12 you'll hear more about that. And the title of his
13 talk is the Epigenetics of Pain.
14 Welcome. Lamarck lives.
15 (Applause.)
16 Presentation – Steve McMahon
17 DR. MCMAHON: Maybe I should start by saying
18 that Pat would hate being at this meeting, I suspect.
19 Genetics, that was not his idea of the way pain
20 research should be.
21 So I was asked to give a list of potential
22 conflicts of interest, which I do here. I would

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1 point out that only one of these interactions has got
2 anything to do with epigenetics, and that's a
3 collaboration with Pfizer, who are doing some
4 ChIP-seq analysis with us. I don't know what you
5 might take from that. It says to me one thing, that
6 the pharmaceutical industry to date have not invested
7 a lot in epigenetics in this area.
8 So that's the title of my talk. Another
9 disclosure is that I'm not an epigeneticist. I'm not
10 a geneticist. I'm a neuroscientist, and I want to
11 give you a perspective on epigenetics from a
12 neuroscientist point of view, and obviously try and
13 draw out some of the relevance to our understanding
14 of painful conditions.
15 But it's worth saying in the audience we've
16 got a couple of proper card-carrying geneticists who
17 are very good to provide a different gloss in some of
18 the discussion.
19 So what I thought I would do is to try and
20 structure my talk like so. I'm guessing that many of
21 you don't have a genetics background, and therefore,
22 it's worth spending maybe a little bit of time just

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1 saying what this epigenetics thing is and how it
2 works. Then I'll say something about what it's meant
3 to do, and then try and get to the main part of my
4 talk of what's the relevance of all this stuff to
5 pain.
6 I'll finish with a little bit of speculation
7 really or discussion about the opportunities to use
8 this knowledge arising in this field of epigenetics
9 to develop strategies to treat pain. And I'll also
10 try and say a little bit about some of the challenges
11 that might be associated with that.
12 So let me start with a definition. What is
13 epigenetics? So you can read this while I'm talking.
14 I stole this from a website put out by a new group
15 created last year, the NIH Roadmap Epigenomics
16 program. And you can see what they said. This
17 definition comes in two parts, but the first one is
18 the kind of formal definition.
19 Epigenetics is an emerging frontier. I mean,
20 the word was coined more than a hundred years ago,
21 but it's still true that it's an emerging frontier in
22 the sense that our understanding is rapidly

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

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

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

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

3 So that's a formal definition.

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

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

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

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

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

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

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

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

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

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

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

22 What's been much, I think, more controversial

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

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

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

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

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

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

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

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

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

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

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

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1 completely confident we understand what they are.

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

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

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

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

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1 of these are pharmacological targets. They're all
2 potentially targets that one could manipulate in
3 order to change these epigenetic processes. And
4 you're going to hear later this morning from Chas
5 Bountra who's going to talk about his efforts to make
6 clean chemicals that will offer a clean set of tools
7 that can be used to target some of these players.
8 So all of this, this complicated set of
9 ideas, give rise to the concept that there's going to
10 be some kind of code, there's going to be a code of
11 epigenetic marking that has a predictable and simple
12 effect on gene function. And from what I've already
13 told you, you'll recognize that that code clearly is
14 going to be very complicated.
15 But on the other hand, I think it looks to me
16 at least as if a fair bit of progress has been made
17 in trying to understand some of the principles of the
18 code. And obviously, through this complexity, if
19 we're going to manipulate the system, we really would
20 need some confidence in there being a code that we
21 can apply in different circumstances.
22 So here's one example, I think, of how these

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1 codes might play out. And what I'm illustrating here
2 is, again, a typical gene with its transcriptional
3 start site, associated promoters, and at some
4 distance some associated enhancer regions, all of
5 which are important in regulating gene function.
6 From what I've already told you, it's true
7 that if these promoters are heavily methylated, then
8 that's associated with a very repressive environment
9 and a failure of transcription, so a repressive
10 state.
11 But there are several other states you can
12 imagine these cells being -- that this gene being in.
13 One would be that you lose some of these repressive
14 marks, and you perhaps get some transcription factor
15 activation, some hypermethylation of the DNA itself.
16 And that leaves the cell. It's still not active.
17 The gene is not active, but it's primed. It could be
18 activated under certain circumstances. And this kind
19 of primed inactive state is often associated with
20 this, this monomethylation of this lysine in histone
21 through.
22 Obviously, there's an active state in which

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1 transcription is on, and in many genes, that's very
2 strongly associated -- perhaps one of the best
3 correlates is with this mark, this acetylation of
4 this lysine K27 in H3 along with trimethylation of K4
5 around the transcription site. So there's a little
6 bit of a code here.
7 But actually, it's also true that there are
8 many intermediate states between primed and active in
9 which the cells are said to be poised. They may be
10 activated by an appropriate series of circumstances.
11 And there may be many different circumstances, many
12 different contingencies which would be specifically
13 be able to active a poised gene. And these poised
14 genes also carry a series of characteristic marks
15 typically.
16 So I think you get some idea that there may
17 be some simple but some rather reproducible patterns
18 of histone marking that can be -- epigenetic marking
19 that can be used to drive particular patterns of
20 transcription. And those patterns of transcription
21 are not just on and off. They're on, off, and maybe
22 if something else is appropriate.

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1 So what's it for? So you've got all of these
2 complicated markings. What does it actually do to
3 cellular function? What are the consequences of
4 having this code?
5 I think again from my perspective, I see sort
6 of three broad areas, which are quite well developed,
7 of more or less interest to us as pain scientists.
8 So one area is in cancer biology. Most tumors show
9 fantastically dramatic alterations in their
10 epigenetic controls. The reasons that tumors are
11 tumorigenic is because of a failure of these
12 epigenetic processes.
13 So the epigenetic control of the cell cycle
14 is a dramatic example of epigenetics in action of
15 what can go wrong. And whilst that's not of great
16 interest to us as pain biologists, it certainly is
17 true that the knowledge that we have, a great deal of
18 it has derived from study of cancer biology because
19 that's where these processes have been marked out.
20 And I suppose there is a cautionary tale there that
21 if we're going to mess around with epigenetic marks
22 or epigenetic processes in order to treat pain

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1 patients, for instance, one ought to be careful about
2 the impact on cell cycle.
3 On the other hand because, as I say, this is
4 a reasonably advanced field, our understanding has
5 come from cancer biology, it's also true that there
6 are a number of drugs in the clinic which are used
7 for the treatment of cancers, which themselves
8 interfere with epigenetic processes. And that's
9 actually quite interesting for us because it means
10 that we have compounds that will be available,
11 potentially available, to test ideas in other
12 systems. So that's one big chunk of epigenetics.
13 A second chunk is that epigenetics are
14 essential for normal development. As I think I've
15 already alluded to, for a long time epigenetics was
16 seen -- even when the mechanisms were not understood,
17 it was seen as conceptually those processes that led
18 to the orderly development of an organism, that the
19 orderly orchestration of cellular events would take
20 you from an embryo to a differentiated organism.
21 That includes a variety of processes.
22 Perhaps the most obvious is in lineage determination.

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1 So these fibroblasts, these Purkinje cells, start off
2 with identical sequence information, genetic
3 information, and yet because of epigenetic
4 processing, they end up expressing different patterns
5 of genes and genes that have completely different
6 forms and functions.
7 So obviously a lot of work has derived from
8 developmental studies, and again, some of our
9 understanding about epigenetic processes have emerged
10 from this field.
11 The relevance of these studies to pain may be
12 not so obvious. There are a couple of intersections
13 between these developmental processes and our
14 interest in pain. Perhaps the most obvious is in
15 pediatric pain states. So one of the things
16 that -- I'm not going to talk about today, but it's
17 at least of interest to some people -- is whether or
18 not pain in development in infancy changes the
19 developmental process and leaves the pain signaling
20 system in a distinct state.
21 That's entirely feasible because I just told
22 you that the orchestration of development requires

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1 the careful integration of epigenetic processes. And
2 so the impact of painful events on those processes is
3 largely unknown.
4 But the third category of -- a good example
5 of what epigenetics are good for is listed here. And
6 that is that there is a growing body of evidence that
7 epigenetics is a way in which -- it's many cells when
8 they are activated or stressed or challenged in a
9 number of ways change their function. And that's
10 certainly a concept that's very familiar to pain
11 neurobiologists.
12 It turns out that that alteration in function
13 is mediated or driven by epigenetic processes in many
14 cases. And so I show one example here that this is
15 using kainic acid. You could actually use a whole
16 number of transmitters and show that hippocampal
17 cells will quite rapidly, within 15 or 30 minutes of
18 exposure to these cellular stimuli, phosphorylate a
19 particular residue. This epigenetic mark is applied.
20 This searing residue is phosphorylated in
21 hippocampus.
22 So there's acute response, this kind of

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1 mechanism of plasticity of cells to environmental or
2 cellular challenge. That plasticity is dependent
3 upon epigenetic regulations in many cases, and that's
4 interesting for us.
5 The other side, though, which I think should
6 be very interesting to us as well, is that these
7 epigenetic processes may provide a kind of cellular
8 memory, a memory of what's happened to those cells in
9 the past. And clearly, a long-term -- so these
10 memories may be very specific for particular types of
11 cells, particular type of stimuli, and obviously for
12 particular genes. That is a very powerful memory
13 mechanism that may have a long-term impact on the
14 functioning of those cells.
15 There's quite a bit of evidence at least
16 theoretically, this can occur. So here's some data
17 from some twins studies. These are identical twins
18 who were studied either when they were very young or
19 a different cohort who were much older. And the
20 experiment is simply to ask whether or not the
21 methylation status of their genome is similar or
22 different.

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1 So their methylation status was determined.
2 And for 3-year-old identical twins, perhaps it's not
3 surprising that they have very similar methylation
4 marks across their whole genome. But for the older
5 twins, it turns out that they have increasingly
6 divergent methylation patterns across their genomes
7 and that the longer they've lived apart, the more
8 extreme these patterns are.
9 Now obviously, we don't know what function of
10 these methylation marks are, but the principle that
11 your cells carry a lifelong history of what's
12 happened to you, I think is enshrined in this simple
13 finding so that these epigenetic marks may determine
14 how you're going to respond to events in the future.
15 But obviously, from a pain perspective, the
16 idea that an individual may have an epigenetic
17 regulation of how, of their propensity, to develop
18 pain states is an interesting idea that I think we'll
19 talk about some more. It's an interesting idea for
20 us as a community.
21 So what about epigenetics? What does it
22 actually do in action? So I picked a couple of

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1 current -- they're not spurious, but an almost random
2 selection of a couple of papers to give you a flavor
3 of the way in which epigenetics is seen to have an
4 impact on nervous system function. And I'll just
5 tell you a couple of very short stories.
6 So here's quite a nice paper that says, well,
7 how is that we reconsolidate memories, distant
8 memories. And obviously, that's some relevance to
9 treatment of patients with traumatic fear memories.
10 And it turns out that a particular HDAC, one of these
11 deacetylases, is critical for this process of memory
12 reconsolidation. There's quite a bit of literature
13 out there looking at how different epigenetic
14 processes contribute to memory formation and
15 reconsolidation in this case.
16 Here's another example. There was a nice
17 study a couple of years ago that was looking at the
18 effects of cocaine addiction in an animal model and
19 very nicely demonstrates this idea that one form of
20 life experiences may predispose you to a different
21 form of response to something else later.
22 So the context here is that repeated -- if

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1 you make rodents addicted to cocaine and then stress
2 them, it turns out that their responses to stress are
3 very much dependent on whether or not they were prior
4 cocaine addicted.
5 It's the combination of the two events that
6 are -- the intersection that are very apparent here.
7 And as this paper says, that histone methylation is
8 essential to leave this mark from cocaine addiction,
9 which will then subsequently affect the individual
10 animal's ability to cope with stress.
11 So the non-addicted animals cope much better
12 to stress than the addicted animals. That's a sort
13 of trivial finding on one level, but a nice
14 epigenetic demonstration of how the memory may
15 operate in practice.
16 My final example, which is a bit closer to
17 home for many of us, and that's the quite basic
18 processes such as nerve regeneration. So peripheral
19 nerve regeneration, as many of you will know, is an
20 orchestrated event, a coordinated event, of some axon
21 growth program that gets switched on.
22 Well, this paper makes a nice case that one

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1 particular epigenetic enzyme, this HDAC5, is
2 important in orchestrating the axonal regeneration
3 program. It's actually important in two ways, which
4 is why I mentioned it. One is that following nerve
5 injury, there is an export of HDAC5 from the nucleus,
6 and that leaves the nucleus at a different
7 transcriptional state because this HDAC5 has moved
8 into the cytoplasm. And that's the nuclear
9 epigenetic part of it.
10 But it turns out that the HDAC5, I mean, many
11 of these enzymes are able to act on not just histones
12 but on cytoplasmic proteins. And this paper provides
13 some nice evidence that HDAC5 was able to deacetylate
14 some proteins in this cytoplasm and contribute to
15 axonal growth that way.
16 So I guess that's just one word of caution
17 that many of the players that we're talking about,
18 yes, we're implying that they have a role in DNA
19 regulation, but they may also have other cytoplasmic
20 roles that we should be careful to unravel.
21 So there's a reasonable literature that the
22 nervous system, the adult mature nervous system, is

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1 using these epigenetic processes in a variety of
2 distinct fashions. And so that begs the question of
3 what does this mean from the perspective of pain.
4 And I think there are two topics I'd like to briefly
5 discuss.
6 Firstly, this one, I think, is always the
7 easiest one, and we have a lot of data on it. I'll
8 show you a little bit of it. And that is that
9 following injury or in some disease states, we're
10 very familiar with the idea that there are
11 pathophysiological consequences that affect many
12 levels of the neuro axons. And those
13 pathophysiological consequences are associated with
14 altered gene expression in many cases.
15 So what I want to tell you is what do we know
16 about the role of particular epigenetic processes in
17 controlling the cellular responses that arise in the
18 face of injuries and presumably contribute to the
19 hypersensitivity associated with those injuries. So
20 that's one topic.
21 The other one, I can't show you a lot of data
22 because there isn't a lot of data, but I definitely

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1 think this is a very intriguing possibility. And
2 it's really the opposite. It's not why do some
3 people get pain after injury. It's really why do
4 some people not get pain; why do most people not get
5 pain after similar injuries.
6 One answer to that question is that the
7 people who get pain happen to have some vulnerable
8 brain, whereas the people who don't get pain have
9 some resilient brain. And the issue is, are there
10 epigenetic processes, perhaps early in life
11 experiences, that predispose us to being vulnerable
12 or resilient? And as I say, I think it's a very
13 intriguing idea, but one for which, as I told you, we
14 don't have a fantastic amount of data as yet.
15 But what about this first thing, what's the
16 evidence that these epigenetic things are any good in
17 coordinating responses to injury? So let me start
18 with here's a little bit of data from a post doc of
19 mine, Ana, who did a next generation sequencing study
20 on DRG neurons after a peripheral nerve injury,
21 frequently used as a persistent pain model, spinal
22 nerve transaction.

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1 What she found was that you get an awful lot
2 of transcriptional dysregulation in DRG. There's
3 nearly 4,000 genes out of 80,000 assessed, which is
4 significantly dysregulated, either up or down
5 regulated. And that really does beg the question
6 what's controlling this altered gene expression, and
7 there are many things that might control it.
8 One that I think has been explored quite a
9 lot is this family that I talked about of HDACs and
10 HATs. So this process, we have a bunch of
11 tools -- they're not fantastic tools. But we have a
12 bunch of tools to interfere with this process. And
13 it's probably the single process that's been most
14 extensively investigated in the pain field.
15 So the idea is that we have a series of
16 enzymes that add acetyl groups to histone residues,
17 and the idea is that that leads to a slightly more
18 relaxed state of chromatin, which will favor
19 transcription. On the other hand, we have a series
20 of enzymes, which deacetylate these histones, leave
21 the chromatin in a more condensed state, and
22 transcriptionally less active in general.

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1 It's a nice system, and I want to talk about
2 it a bit because we do have a whole bunch of slow
3 molecule inhibitors that are known to inhibit HDACs
4 and thereby prevent this deacetylation to lead, we
5 think, to a more acetylated state and perhaps a more
6 transcriptionally active state.
7 So here are the little list. We have in this
8 class of HDACs already quite a number of distinct
9 HDACs falling into several subgroups, and we have a
10 number of molecules that are able to target these
11 different HDACs with varying degrees of specificity.
12 And I think it's worth saying that I don't think any
13 of these compounds have ever been formally assessed
14 for their non-HDAC activity, but they almost
15 certainly are not clean specific compounds. But
16 they're what we have. And as it happens, some of
17 these have reached the clinic in the treatment of
18 pain cells.
19 So we have a number of tools and is one way
20 in which we can ask as pain neuroscientists whether
21 this family of enzymes is important in its ability to
22 affect pain states.

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

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

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

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

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

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

11 We did another experiment with Andrew Rice's
12 group. We developed this model, and it's published
13 now, of d4T-induced neuropathy, a chemical neuropathy
14 rather than a traumatic nerve injury one. And again,
15 pretty much an identical paradigm and an identical
16 result. Again, this was a really blind experiment.

17 The two labs were not in any contact at all.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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1 images from the Allen Brain Atlas. I told you there
2 were three Dnmts, 1, 3A and 3B, that is supposed to
3 regulate methylation. This one is supposed to
4 maintain -- is responsible for the maintenance of DNA
5 methylation, these two for the more kind of plastic,
6 inducible forms of methylation.
7 You can see these are obviously developing
8 animals, but at P4, the DRG is very rich in Dnmt1 and
9 3A, and there's almost no 3B. That's consistent with
10 our own sequencing data showing rosy values here. In
11 these neuropathic lesions, this methylase does go up,
12 and that's associated with the increased methylation
13 that's produced.
14 As I say, I think we've got good tools to
15 study this process now. So you're going to hear
16 later from Chas Bountra, whose group have made very
17 specific DNA methylase inhibitors, Dnmt inhibitors.
18 And here's just some data we've got using their
19 compound. Single intrathecal dose leads to a fairly
20 long-lasting alteration in methylation.
21 There are also transgenic animals coming
22 along. There's a floxed Dnmt3A/3B animal. There's

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

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

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

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

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

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

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

22 That's an attractive model for quite a number

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

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

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

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

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

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

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

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1 hundreds of epigenomes.
2 So here's a nice paper that appeared last
3 year that simply took a whole bunch of different cell
4 types, and you can see what they are. Some of them
5 were -- this is a tumor cell line. These are primary
6 cells. These are sperm. And they did DNA
7 methylation across the whole genome and then see how
8 these cells clustered.
9 The important point is a principal component
10 analysis. They're two-class, but they're all very
11 distinct. So the epigenomes of every cell type is
12 likely to be different.
13 Well, that's a problem because how are we
14 going to -- and where are we going to look? The
15 problem now is which cell are we going to pick now as
16 being pain relevant in our system.
17 As it happens, just as an aside, a bit of a
18 plug, one of the things we spent the last year doing
19 is trying to play with FACS sorting and magnet
20 sorting to see if we can get pure populations of
21 cells so that we can do some of these epigenetic
22 studies.

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1 So one example is illustrated here. If you
2 just disassociate DRG neurons and put them in a dish,
3 it turns out that only about 25 percent of the cells
4 are neurons. The rest are satellite cells and other
5 cells. You can purify those with magnetic sorting
6 and get a 99 percent pure DRG population. We can do
7 the same thing with spinal microglia. We can do the
8 same thing with transgenically marked cells that
9 carry fluorescent tanks.
10 So I do think these approaches will give us
11 an extra lever to try and identify epigenetic
12 processes in a cell-specific way.
13 So I think from what I've been saying that
14 all of these different mechanisms do look attractive
15 from pain biologists. Of course, these mechanisms
16 are at play in many, many cells and for many
17 different purposes. So as usual, we're going to have
18 a real problem perhaps in identifying those processes
19 that may be the most specific.
20 On the other hand, I know industry's been
21 very keen to look at biomarkers. You really would
22 think that simple epigenetic evaluations, looking for

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1 particular marks of particular residues in particular
2 cells, that may be a very powerful way of identifying
3 biomarkers for how people are responding to
4 particular disease states or how they're going to
5 respond to a future disease state.
6 I have seen that this -- I mean, I thought
7 I'd finish with -- I saw this in the latest edition
8 of Nature Neuroscience. I see what's become very
9 trendy has been to try and combine some of these
10 genome-wide analyses with functional imaging.
11 So here's a review paper, this is done not
12 with epigenetics but with genetics, looking at
13 efforts to try and combine GWAS studies with
14 functional brain imaging either to ask whether there
15 are particular variants that are associated with
16 particular forms of brain activation pattern or
17 associated with particular structural changes in the
18 brain.
19 I think that this is an immensely complicated
20 procedure, but there's a real opportunity to do
21 exactly the same with epigenetics and say can we have
22 epigenetic marks that predict differences in

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1 behavior.
2 I'd like to finish by just thanking the
3 people that have done the work. This is a group at
4 Guy's Hospital in London. Most of the epigenetic
5 experimental data I've shown was generated by Fran
6 and Maggie. So thank you very much.
7 (Applause.)
8 DR. BASBAUM: We have time for some
9 questions.
10 DR. MCMAHON: It's you he's talking to.
11 DR. BASBAUM: Can you actually reverse, or
12 would you predict that you can reverse an epigenetic
13 change or if you can prevent --
14 DR. MCMAHON: So there's a little bit of data
15 there. The Wang study I showed -- yes, and that's
16 whether or not these things, whether you can prevent
17 them, which I think a lot of the interventions have
18 tried to do and done successfully, or whether you can
19 allow a change to become established and then try to
20 reverse it by blocking some epigenetic process.
21 So that's been done a few times. The Wang
22 study actually produced a nerve injury, allowed some

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1 transcriptional change to get going for three days,
2 and then gave the methylase inhibitor. So they were
3 able to reverse a partially established state.
4 We did the same thing with the HDAC
5 inhibitors intrathecally, and we couldn't. So once
6 we established a pain state, then the HDAC inhibitors
7 did nothing in our hands or at least the ones we
8 used.
9 Actually, it's another reason -- although
10 that's -- it obviously has a bit of an impact for
11 therapy. It also convinced us that these drugs were
12 not just having nonspecific actions on the pain
13 signaling system because the same drug and the same
14 dose was ineffective in those conditions.
15 DR. GLORIOSO: So I thought it was really a
16 wonderful talk. If you can separate different nerve
17 cell populations for DRG in which there's chronic
18 pain going on, have you tried to look at individual
19 subpopulations to see if particular ones have genetic
20 changes or epigenetic changes and others do not?
21 DR. MCMAHON: That's the idea. So we
22 struggled a bit to get pure subpopulations. I don't

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1 think we're alone in this. I think you'll see a
2 whole spate of publications coming out using these
3 techniques.
4 So yes, you clearly can. You can identify
5 subpopulations, and then you can genotype those. You
6 can epigenotype them. There's a sensitivity issue,
7 but that's -- I think maybe Jordana might say
8 something about the technical difficulties of doing
9 some of these experiments, but theoretically, yes.
10 DR. GLORIOSO: Well, it raises the
11 possibility that you can deliver a gene like an HDAC
12 directly to the correct subpopulation and perhaps
13 change that population in particular.
14 DR. MCMAHON: That would be nice. I mean,
15 you're right, targeting agents to particular cells,
16 there's a little bit of precedence for that. But
17 that's non-trivial as well, of course. But yes,
18 theoretically.
19 DR. MAIXNER: Bill Maixner, UNC. Stephen, is
20 there any emerging knowledge about the type of
21 environmental exposures that will lead to the
22 different regulatory pathways for epigenetics? Are

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1 there specific environmental factors that may lead to
2 alterations of methylation versus a histone
3 modification?
4 DR. MCMAHON: Yes, I mean, it's certainly not
5 been done comprehensively. The one manipulation that
6 everyone agrees has a profound effect on brain
7 development in a number of ways is early stress. So
8 stress in different forms is definitely a major
9 dictator. Whether or not -- I mean, it's
10 interesting.
11 The hypothesis is that that may predispose
12 people to some forms of disorder like fibromyalgia or
13 irritable bowel. That's really to be formally
14 established. But if that was true, then it would
15 imply there was some specificity in the system. But
16 that hasn't been demonstrated to date.
17 DR. FARRAR: John Farrar, University of
18 Pennsylvania. I'm going to demonstrate my naiveté
19 when it comes to this whole topic area, but it's an
20 amazing system. The question, I guess, is what
21 controls this system and why would certain kinds
22 of -- following up on Bill's question, why would

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1 certain types of environmental deprivation or other
2 things lead to certain processes being turned on or
3 turned off?
4 DR. MCMAHON: But they do. I mean, that much
5 we know.
6 DR. FARRAR: Right.
7 DR. MCMAHON: So the range of things that
8 we're interested in that can do it are any
9 neurotransmitter, nerve injury, BDNF. I mean,
10 there's lots of the players we're familiar with.
11 The cocaine story, the suggestion there is
12 that an increased transmission via BDNF being
13 released and acting on TrkB is the trigger for the
14 epigenetic changes.
15 So many of the common things that we're very
16 familiar with lead through second messenger systems
17 to the recruitment of these epigenetic processes. I
18 didn't really talk much about that, but yes, it's not
19 magic actually, yes.
20 DR. FARRAR: But I agree. The question is
21 that I would assume that the different stimuli you
22 just spoke about led to slightly different epigenetic

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1 changes. Is there an underlying process by which
2 certain ones lead to second ones?
3 I think you're suggesting that there's
4 connections, that there's transmitters that change
5 them in specific ways. But I'm just wondering if
6 there's any work that's looked at, why it is that
7 cocaine exposure leads to a certain set of epigenetic
8 changes.
9 DR. MCMAHON: So there is -- although I think
10 specificity is going to be an issue, there is quite a
11 lot of specificity in the system. So I showed you
12 one little bit of from the Allen Brain Atlas that one
13 of the DNA methyltransferases is just not expressed
14 in DRG neurons. And we know that some of the other
15 players are tissue specific in their expression.
16 So we do have a bit of something rolling for
17 us to start with. There's some specificity in the
18 system that we might be able to capitalize on.
19 But otherwise, no. It's going to be like all
20 of the other things we've been studying. What is the
21 unique combination of events in this patient or under
22 this model at this condition that trigger the change?

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1 DR. PORRECA: Frank Porreca, University of
2 Arizona. Wonderful lecture, Mac, as always.
3 I just had really two questions. The first
4 is the -- I wonder if you could comment on the
5 temporal events that might be seen in some of the
6 experimental models. I mean, if you looked at these
7 profiles at a certain time point, is it what you
8 would expect to see many months later when things may
9 be changing? There is some evidence that there are
10 changes that occur quite late that may have
11 potentially stronger correlates to the human
12 condition. That's one question.
13 The second thing is, any attempts to separate
14 the injury from the so-called pain that might occur.
15 I mean, is that separable? Can you actually identify
16 and link these profiles that you've been looking at
17 in the spinal cord, for example, or in the DRG, to an
18 expression of a pain state, or is it just the
19 consequence of an injury that doesn't necessarily
20 link to the pain?
21 DR. MCMAHON: So two good questions. The
22 first one, I think Jordana's got some data on

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1 stability of these changes over time, so maybe she
2 can address that.
3 I mean, although one of the attractions of
4 this system -- in the pain field for a long time,
5 we've been looking at mobilization of second
6 messages, sensitization over minutes. I mean, these
7 processes are distinct, that they leave a mark that
8 is stable. I mean, it's around for a long period of
9 time, days, weeks, months, potentially years.
10 So they definitely have a more inherent
11 long-term course of action. How stable they are,
12 there's been a few studies, not so many in pain.
13 Your other question was?
14 DR. PORRECA: [Inaudible - off microphone.]
15 DR. MCMAHON: Oh, to pain. Yes, I know, so I
16 did sort of breeze over a bit. Yes, you're
17 absolutely right. In nearly all of these studies, we
18 don't have causality. We have two lots of phenomena,
19 and we'd really like to demonstrate what's causing
20 what.
21 I mean, it's possible, but that hasn't really
22 been done to such an -- and I should say, I focus on

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1 DRG and spinal cord because I know the biology there,
2 but you'll hear later that there -- these epigenetic
3 processes are at play up and down the neuro axis in
4 pain states as well. So it's not just in peripheral
5 tissues.
6 DR. BASBAUM: Steve, thanks very, very much.
7 (Applause.)
8 DR. BASBAUM: Thank you. That was a great
9 start, and it's my pleasure to introduce Laura Stone,
10 who is another good friend for many years.
11 Laura did her original work at the University
12 of Minnesota, went to OHSU, and then went into
13 industry. And there's a theme here that we're
14 hearing, but then came back to academia and is now on
15 the faculty in the dental school at McGill
16 University.
17 Laura's original background was largely in
18 pharmacology, spinal cord primary afferents, and then
19 more recently in the last few years, has been working
20 in a really provocative model, which I think is one
21 of the most intractable problems both in the animal
22 and clinical model, namely, back pain. And today,

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1 she'll tell us about her work on SPARC and back pain.
2 Welcome, Laura.
3 Presentation – Laura Stone
4 DR. STONE: So to start with, thank you for
5 inviting me. I don't have any disclosures, but a lot
6 of people I want to thank and a lot of funding
7 agencies to thank listed here. And I want to make a
8 comment about the funding from Pfizer was part of
9 a -- Pfizer Canada has these neuropathic pain
10 research awards, so it's a peer-reviewed open
11 competition. It's not a contract or in any
12 way -- they don't have any say in what we actually
13 do. They just fund it.
14 So everybody knows that back pain is probably
15 the most common chronic pain condition and one of the
16 hardest to treat. And so much research has been done
17 in chronic back pain, and we've pretty much gotten to
18 this point where we think it might be something about
19 the brain and there might be some stuff happening in
20 the spine. This is the current status of this field.
21 So we have to start somewhere. Maybe make a
22 little bit more progress than this. The vast

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1 majority of cases of chronic back pain, we cannot
2 find the cause. It's unknown. It might be something
3 in the spine or in a ligament or in the muscle, but
4 we can't find it.
5 There are some cases, though, where the back
6 pain seems to be linked to problems in the spine,
7 disk herniations, disk degeneration. So this is at
8 least something that we can study. It certainly
9 doesn't represent all back pain patients, and some of
10 the correlations between back pain and the spinal
11 pathologies are weak. But we can see this. We can
12 measure it. We can induce it. So it's something
13 that we can at least begin to study.
14 There are many ways that disk degeneration
15 could end up producing pain, and this is just a
16 schematic attempting to outline a couple of these.
17 So here's a healthy normal disk. Here's a
18 degenerating disk, and it's narrower. And then we
19 have some bulging in herniated disks.
20 A lot of mechanisms are going on during this.
21 So we have local inflammation in and around the area
22 of the injured disk. We have nerve compression, but

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1 also, there could be compression of DRG neurons or
2 spinal cord. And the compression actually also
3 happens here when there's just degeneration. It does
4 not require herniation.
5 The inside contents of these disks are just
6 loaded with pro-nociceptive and pro-inflammatory
7 compounds. If you take the inside of a disk and drop
8 it on a nerve, you will induce neuropathic pain
9 symptoms for several weeks in animal models, so just
10 the inside of these disks is really nasty. So if
11 they start to leak or if they herniate and nerves
12 come in contact with these inner contents, that's a
13 very probable source of pain.
14 Finally, there's now evidence that as disks
15 degenerate, they're normally not really innervated
16 because we don't want nerve fibers to normally be in
17 contact with all these nasty things inside. But as
18 they degenerate, the nerve fibers start to grow in
19 deeper pathologically. So now you have nerve fibers
20 that are inside the disks that are experiencing a
21 chemical environment and mechanical stresses
22 activating nociceptors where they should not be

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1 there. So normal movement, normal physiology now,
2 might activate nociceptors and cause pain.
3 This is an example from a disk taken from a
4 back pain patient just showing an example of
5 innervation. This was found quite deep in the disk,
6 and you can see there's a blood vessel. And then
7 this is PGP staining for nerve fibers that were
8 growing deep into the disk along that blood vessel.
9 Sometimes we also see free nerves deep inside disks,
10 but they're not supposed to be there.
11 The question is how are we going to study the
12 relationships between back pain and disk degeneration
13 and innervation without an animal model because we
14 have some problems dealing with human models. Like,
15 you can't always get the disks. We can't have
16 control disks. Back pain is very complicated.
17 There's all of the emotional and cognitive factors in
18 patients. So we really need an animal model to start
19 to kind of look at the physiology of this.
20 So we started just looking in the literature
21 to see if there's anything out there, any animal
22 models of disk injury or disk degeneration that we

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

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

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

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

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

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

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

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

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1 going to activate those fibers, I should say.

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

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

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

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

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

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

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1 So we think that what we're seeing is
2 actually a resistance to having this kind of stretch
3 along the spine.
4 Measuring radiating pain in one or both of
5 the hind paws is much easier. We have many evoke
6 tests for that. So this is an example of cold
7 sensitivity using the acetone test where null wild-
8 type mice don't respond much to the acetone test, but
9 the SPARC mice have an elevated responsiveness.
10 This is probably the most consistent
11 phenotype that we see. And it's interesting. It
12 gets worse with age, and at some point, the wild-type
13 mice catch up. And if you're a really old wild-type
14 mouse, you also have cold hypersensitivity. And
15 there's a joke in there about Florida and Canadians.
16 (Laughter.)
17 DR. STONE: Having this sort of chronic
18 progress model is going to allow us to do a lot of
19 different types of studies than we would normally do
20 in induced models, where it's sort of an all or
21 nothing thing. And what I'm going to just show you
22 today is one example from an exercise study because

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1 it really highlights the value of having this sort of
2 progressive model. But there are many other things
3 that we could talk about offline.
4 So in this experiment, this is the
5 intervention. It's a little plastic wheel. It costs
6 \$3. We put it in the home cage, and that's the
7 experiment. Mice love it. They run on it all the
8 time. The control group is the little plastic wheel
9 with a screw placed in it so that it doesn't spin
10 anymore. So they can still play in their hut, and
11 they can still sniff it and chew on it and do what
12 mice do. But it doesn't spin, so they don't get that
13 exercise component.
14 So what we found is the following. So now
15 I'm just showing the acetone behavior. So this is a
16 radiating pain test, and in wild-type animals, we
17 just had some cages of wild types as controls. And
18 you can see at the baseline, all of the SPARC mice
19 have got elevated cold sensitivity that we talked
20 about.
21 This dotted line is the SPARC control where
22 their wheels didn't spin. And here's what happens in

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1 the SPARC mice that had spinning wheels. It takes
2 one to two months, but the cold allodynia completely
3 reversed.
4 The first question that anyone has when you
5 look at exercise related analgesia is, is this an
6 acute opioid release effect. So we reversed it by
7 just taking away the spinning wheels. In fact, we
8 just put a screw in them so they didn't spin anymore
9 in some of the groups.
10 What you see here is the ones that continued
11 to have spinning wheels, they kept down there, but it
12 took a little while, actually a couple months, of not
13 exercising before the phenotype completely reversed.
14 We weren't really sure how far to go out
15 here, so we tested it at one month after the wheels
16 were taken away, and there was still the therapeutic
17 benefit. And we didn't want to publish a paper that
18 says that you only have to exercise like for a couple
19 weeks and then have it last forever. So we kept
20 testing them and testing them, and it did finally
21 reverse.
22 The amazing thing here -- and I'll get to the

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1 epigenetic part in a minute -- was that it changed
2 the disk pathology. So looking at the disks
3 afterwards, we can see looking at wild-type control
4 versus the SPARC control, there's a loss of disk
5 height normally -- that's part of the
6 phenotype -- that was partially reversed by the
7 exercise.
8 In addition, there is a recovery of
9 pathological innervation. So this difference is
10 pathological innervation in SPARC compared to
11 wild-type. This is what happens in the exercise. So
12 we've actually, just by a behavioral modification,
13 reversed some of the pathology that's potentially
14 associated with back pain.
15 SPARC is a model -- we think it's a model of
16 accelerated aging. But we wanted to kind of get a
17 little bit more how this would relate to normal
18 aging, and the first thing that we did to address
19 this is to ask the question is what happens to SPARC
20 in normal animals as they age because the mice get
21 old enough, they start to have disk degeneration.
22 They start to have signs of pain.

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1 This is a SPARC mRNA expression, and this is
2 how -- this should be months. This is not weeks.
3 These are our 15-month mice. With time, expression
4 of SPARC goes down as we age. So essentially as we
5 all get older, we're becoming SPARC null mice.
6 To quantify that, we're able to measure disk
7 height by X-ray. And I just really like this figure
8 because you can see here by histology and herniation.
9 And look at the loss of height in the space here,
10 whereas here's a nice, healthy disk, and there's a
11 nice space in there. So we can measure that space by
12 X-ray and get an index of the degree of degeneration
13 of that disk.
14 So in SPARC mice, we know there's a loss of
15 disk height, and now also in aging mice, we see at
16 the three ages, and this is also months, that there
17 is a progressive loss of disk height in normal aging.
18 And this is what we see in humans.
19 We also looked in innervation, and we see a
20 increase in innervation with aging. And that's been
21 quantified. And what's interesting here is -- so the
22 SPARC is the red ones. Eventually, the wild types

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1 here as they get old enough, they have hyper
2 innervation as well. It just takes longer. So the
3 SPARC really is just accelerating this process.
4 Finally, the behavior. So this is the SPARC
5 data that I showed you before, and we have very
6 similar phenotype for both the axial pain test and
7 the radiating pain test. Here it's cold. And an
8 interesting thing is that in the SPARC mice, we never
9 see mechanical hypersensitivity. It's only cold.
10 And that's exactly what happened in the aging mice as
11 well. So we have no idea why that is, why we would
12 get cold and not mechanical, but it's really
13 intriguing.
14 Anyway, so what is the mechanism controlling
15 SPARC expression is the next question. And at the
16 time that we were doing this work, it was already
17 known from the cancer literature that epigenetic
18 modulation of SPARC was important in a lot of
19 different disease conditions, in cancers and a couple
20 of other things, too. And the methylation in
21 particular was very well understood.
22 So this is a slide from Denk & McMahon

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1 basically to remind you about epigenetic mechanisms.
2 And I'm going to focus here on DNA methylation
3 specifically as the mechanism. And the way that -- I
4 know we already covered this in the last talk, but
5 just to kind of reiterate, that what happens -- so
6 these are the chromatin, and this is where the
7 transcription factor normally binds.
8 If you have methyl groups here, your
9 transcription factor can't bind, or you get methyl
10 groups there and a bunch of other stuff binds that
11 causes that chromatin to inactivate. But either way,
12 the net effect is that instead of the gene being
13 made, the gene is not made. So more methylation
14 means less gene expression, as is the general rule.
15 This is what the mouse SPARC promoter looks
16 like, which is kind of boring. I realize that, but I
17 just wanted to point out that there's multiple sites.
18 This is that CpG island that Dr. McMahon talked
19 about. So there's multiple sites here where we can
20 ask the question is the methylation here changed in
21 aging mouse disks.
22 We hypothesize that there would be increased

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1 methylation because we have a decrease in the RNA.
2 So that's basically what we got. So in this graph,
3 each of these triplets is a different methylation
4 site. And the Y axis is the percent methylation at
5 that site, and then within each triplet, we have
6 three different ages.
7 It's not at all sites, but multiple sites in
8 the CpG island had increased methylation as the mice
9 aged, which is consistent with the reduced expression
10 that we see.
11 We were curious if we could use a drug to
12 target this and see if that could rescue the reduced
13 SPARC expression. So in this case, we used the drug
14 5-HDAC, where that was also, fortunately, introduced
15 in the previous talk. So now these are all old mice.
16 They're 1-year-old mice in this case.
17 In the drug-treated, in several of the sites,
18 the drug did cause some demethylation. So it did
19 that, change RNA expression. And it had a huge
20 effect on RNA expression.
21 So this is now SPARC expression in the disk.
22 This is untreated, and this is treated. So we can

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

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

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

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

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

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

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

22 So anyway, we have disks -- so then these

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

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

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

18 DR. BASBAUM: Couple more minutes.

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

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

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

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

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

22 So we have this sort of plasticity in this

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

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

11 So we decided to go really chronic because we
12 wanted to know -- we thought really chronic would be
13 the most clinically relevant. So we started there.

14 And what we have to do now, of course, is back up and
15 look at when these changes occur. Is it one day
16 after nerve injury? Is it two weeks after injury?
17 Is it six months after injury?

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

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

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

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

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

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1 But the real question is, well, what happened
2 to the brain? Did it reverse the pain-related
3 epigenetic changes in the brain? And it really did.

4 So this is the change that you saw before basically
5 in just normal and nerve injured, and here's what
6 happens to that nerve injury group after several
7 months in environmental enrichment.

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

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

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1 lights.

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

9 I suppose I'm done.

10 (Laughter.)

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

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

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

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

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

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

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1 like the swimming, and they don't show signs of
2 depression in the other tests. But the tail
3 suspension assay is not the only thing that suggests
4 that there's a resistance to gravity and stretching
5 of the spine.
6 We've also shown it in the grip test where
7 they have to grab on for their tail. They really
8 don't like having that kind of stretching there, and
9 there's a couple other kind of functional tests that
10 we can talk about offline. So it's not the only
11 evidence for that we have. Thank you.
12 (Applause.)
13 DR. BASBAUM: Thank you very much.
14 DR. MAIXNER: Well, it's my pleasure to
15 introduce our next speaker. Dr. David Clark from San
16 Francisco is a professor in the Department of
17 Anesthesiology at the VA Hospital and at Stanford as
18 well.
19 I have followed David's work for some time
20 with sort of co-interest in opioid hyperalgesia, some
21 of the underlying mechanisms associated with opioid
22 hyperalgesia. I think David has been a luminary in

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1 pointing out some of the genetic pathways in this
2 area.
3 I know David has recently switched into other
4 areas related to the neuroimmune system and
5 neuropathic pain. So he has a wealth of knowledge to
6 pick from today, to speak to us today on this topic
7 of genetic and epigenetic contributors to persistent
8 pain.
9 David.
10 Presentation – David Clark
11 DR. CLARK: Well, thank you very much. I
12 appreciate being asked and invited to speak today. I
13 have no disclosures. I'm not going to be
14 recommending that you use any particular drug for any
15 particular purpose. All of the experiments that I'll
16 display were supported by federal funds, and I have
17 no particular industrial ties to any of this work.
18 My colleagues and I are interested, in part,
19 in understanding data like these. So what you see
20 here are some data that were collected by a
21 colleague, Ian Carroll. And what he did was he asked
22 patients after their surgeries how long they were

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1 having pain. So he had a phone bank of students, and
2 they would call every day, even weekends, no kidding,
3 and ask are you having any pain from your surgery.
4 And he did this for months after the operations.
5 What you see is that you have a gradual
6 decline in how many patients are saying yes. It's
7 probably a little slower than you might have
8 expected, but it does come down.
9 Two things to point out, though. One is even
10 though most people will experience resolution of
11 their pain after maybe a month or so, many are
12 experiencing pain way out here after many, many
13 months. And if you are a person who happened to have
14 relatively high postoperative pain scores, it's all
15 the more likely you will have that persistent pain.
16 Persistent pain after surgery, it's a
17 problem. It's a problem for rehabilitation. It
18 leads to very high healthcare expenditures. It's
19 very difficult to deal with in clinics, and we wanted
20 to understand some of the factors that might
21 contribute to data like those. So we have most
22 recently turned to epigenetics as a group of

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1 mechanisms that might help us understand that
2 problem.
3 We've had some marvelous discussion of
4 epigenetics. The particular subfield of epigenetics
5 that we chose to look at was histone acetylation, as
6 was very carefully laid out this morning. Basically
7 what you have is a process where histone
8 acetyltransferases are putting acetyl groups on
9 lysine residues on the tails of histone proteins.
10 That leads to a relatively open chromatin structure.
11 There's more transcription. There's more message for
12 the genes around. If you have a deacetylase come
13 along, you have recondensation of that material and
14 less transcriptional activity.
15 Of course, there are families of enzymes that
16 are governing these processes. And the only thing
17 I'm going to mention is that most of the tools we
18 have available currently to pharmacologically address
19 these are really not so good, especially over here in
20 the area of blockers of histone acetyltransferases.
21 We will use anacardic acid and curcumin as kind of
22 probe compounds, not terribly selective.

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1 The pharmacology for the deacetylase
2 inhibitors are much better developed and become even
3 more so, probably as was mentioned this morning
4 already, because of the interest in these compounds
5 as being useful in the control of cancers.
6 So we're interested in incision and
7 operations. So we took our mice, and we made
8 incisions in a hind paw. And we asked what happens
9 if we go messing with histone acetylation in those
10 animals.
11 So we use -- histone deacetylase inhibitors
12 should prolong the -- it should increase the level of
13 transcription of the genes controlled by histone
14 acetylation in those animals. And sure enough, what
15 we have is a much prolonged period of mechanical
16 sensitivity after those incisions. We're giving the
17 drugs here starting at the time of the incision and
18 continuing for four days after the incisions are
19 made. Thermal sensitization is not touched.
20 If we do the converse experiment with an
21 acetyltransferases inhibitor, what we have is a
22 tendency to reverse or make more rapid the recovery

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1 of mechanical sensitization after those incisions are
2 made. Again, thermal sensitivity isn't touched.
3 I see Dr. Porreca in the audience. He has
4 gotten us all to think a little more about
5 spontaneous pain and the ongoing aversive quality of
6 the incision that might best be looked at using
7 something like conditioned place preference. And
8 though I didn't bring the data along today, I can
9 tell you in the conditioned place preference model,
10 if you block the histone acetyltransferase, you will
11 also reduce that evidence of spontaneous pain, if I
12 might call it that for the moment, that the animals
13 seem to be displaying.
14 We did, however, move into a model of latent
15 pain sensitization in these animals. Why would we do
16 that? Because this latent sensitization or
17 hyperalgesic priming, as it's sometimes referred,
18 might reflect one of the types of processes
19 reflective of persistent pain. This might be
20 something that sets the animals up, perhaps the
21 humans up, for persistent pain.
22 So in this particular model, we make our

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1 incision back here with or without the epigenetic
2 agents, and we let the animals recover for a couple
3 of weeks. And two weeks, they are back to their
4 baselines when you look at their nociceptive
5 thresholds.
6 Then we would inject a little bit of PGE2,
7 which normally leads to a couple of hours of
8 sensitization in the hind paws, and we ask what
9 happens. Well, what happens is if you make an
10 incision back here, even using a vehicle, the period
11 of time the animals show sensitization after
12 injecting the PGE2 is much prolonged, days to recover
13 rather than hours to recover.
14 We did not, as we thought we might have, make
15 it worse by virtue of giving the deacetylase
16 inhibitor, that's SAHA, give the deaceyltlate
17 inhibitor at the same time that we made the incision,
18 so the same level of enhanced sensitization.
19 However, if we had given the animals a
20 histone acetyltransferase inhibitor, here we used
21 anacardic acid, then we see that we were able to
22 block some of that sensitization two weeks hence. So

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1 we feel that perhaps there's a reason to suppose
2 there are epigenetic processes leading to that more
3 persistent sensitization in the animals.
4 Moving on from simply looking at nociceptive
5 and pain-related matters, we are more and more asked
6 to determine what's happening with animals
7 functionally not only because that might give greater
8 basis for any conclusion we draw about what our drugs
9 are doing, but also I'd point out, in the clinical
10 world, restoration of function is a very important
11 goal. And I think if you look at things like impact
12 guidelines and what you should be looking at when you
13 judge the efficacy of an analgesic, you'll see
14 functional goals included as well.
15 So we decided since we're making hind paw
16 incisions, our function would be gait. We did our
17 gait analyses using a video-assisted system. This
18 has a transparent moving treadmill. You take little
19 videos, and you let the computer spit out lots and
20 lots of parameters related to gait. If we do this in
21 our incised animals, we find nine parameters.
22 They're listed on the side here, which are made

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1 abnormal in the setting of incision.
2 This is two days later, sort of 1 to 3 days
3 is where we see this. If curcumin, here we're using
4 it as a probe compound to block histone
5 acetyltransferase activity. If we include that drug
6 at the time of incision and for the first two days
7 afterwards, in fact, we normalize 8 of 9 of those
8 parameters.
9 So perhaps we can even normalize function if
10 we can block some of these changes. That would be
11 our hope.
12 So I'm showing you some pharmacological data
13 and some behavioral data such as they are, implying
14 there could be some epigenetic changes we might be
15 interested in, in understanding persistent pain after
16 incision. But we haven't really talked about histone
17 acetylation and evidence specifically for it.
18 So remember, histone acetylation is one of an
19 array of things that can happen to histone proteins
20 to change gene transcription. Acetylation occurs in
21 all kinds of residues on these tails. This is the
22 ninth lysine residue on the H3 histone subunit, and

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1 I'm going to call that H3K9 just for short. It is
2 only one of many, but a particularly well studied
3 residue that controls things like memory and drug
4 addiction and has really quite a history at this
5 point of being associated with plastic changes in the
6 central nervous system.
7 So we chose to focus in our initial
8 experiments on that particular derivative, H3K9, and
9 we began then by simply staining spinal cord tissue,
10 asking do we find more acetylated H3K9 in the dorsal
11 horns of the spinal cords. And the answer is we do,
12 and most of that -- and if we include a deacetylase
13 inhibitor, one would make incisions, the level of
14 acetylated histone is that much higher.
15 Most of that turns out to be in neuronal
16 cells but not all of it. We haven't made careful
17 study of exactly what the proportions are in this
18 model of glial versus neural. It's way weighted
19 towards the neuronal, but I'm not going to exclude
20 non-neuronal cell types from being involved in these
21 phenotypes.
22 We have now some evidence of acetylation, and

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1 as the previous speakers discussed, you might want to
2 have a candidate gene or try to understand what it is
3 that particular epigenetic change is altering.
4 Ultimately, this should be a change in transcription
5 level.
6 So what we did was we did a big series of
7 array experiments, which were long and painful. And
8 the outcome is when we made incisions, the animals
9 included histone acetyltransferase inhibitors and
10 deacetylase inhibitors and tried to probe which genes
11 are likely epigenetically regulated in this model, we
12 came up with a list of about a dozen or so that we
13 were pretty convinced were altered epigenetically in
14 the model.
15 We chose to use as our sort of probe set of
16 genes this pair. This CXCR2-CXCL1, you'll recognize
17 as a chemokine signaling receptor and ligand pair.
18 Why did we do that? Well, one, we found the pair,
19 and we found that kind of intriguing that both would
20 be regulated under similar conditions. They're well
21 established to play roles in nociceptive
22 sensitization in other models, both centrally -- and

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1 that comes -- and these are recent papers that were
2 published looking at neuropathic and inflammatory
3 pain.
4 Even in the periphery -- and we'll get to
5 some peripheral stuff as well -- where CXCR2-CXCL1
6 seem to be operative, what was very encouraging was
7 others had shown these already to be epigenetically
8 regulated genes. So that was just too good to pass
9 up. And we proceeded by looking at CXCR2.
10 So we took our animals, made incisions,
11 included the deacetylase inhibitor. And sure enough,
12 we had one message for CXCR2, and that translated to
13 increased protein levels as well. Where is that
14 stuff? CXCR2 is primarily in neurons. That is where
15 we expected to find it, and that's where, in fact, we
16 did find it. And those particular neurons expressing
17 CXCR2 also had, for the most part, elevated levels of
18 acetylated H3K9. So we saw that sort of come
19 together.
20 That would be the receptor side of the story.
21 What about the ligand side of the story? Just to
22 broaden this a little bit, CXCL1 is one of the

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1 endogenous ligands. CXCL2 is another. I recall that
2 these things, these chemokines, are named based on
3 the number of spacing residues between those,
4 crosslinking the backbone of the chemokine structure.
5 So we're dealing with the CXC chemokines here.
6 When we went ahead and made the incisions and
7 looked at the spinal cord tissue, it was really only
8 for CXCL1, which also goes by KC and about a half a
9 dozen other names as well. But KC or CXCL1 is
10 elevated, and it's pushed up just a little more, we
11 found, not maybe as much more as would have made me
12 say dramatically, but a little more if you include
13 these deacetylase inhibitor.
14 No such change in our hands for the other
15 endogenous ligand.
16 Now, we have some pharmacological data, and
17 we've measured some message levels and things. But I
18 haven't shown you yet that this has anything to do
19 with histone acetylation. Does histone acetylation
20 control the transcription of those genes?
21 To get at that question, we used what's
22 called a ChIP assay. That's chromatin amino

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1 precipitation, and that is conducted as follows. You
2 crosslink DNA to histone proteins. You fragment the
3 DNA somehow. You can use sonication. You can use
4 other means. You can use enzymes. You then
5 precipitate based on the particular, in our case,
6 histone derivative that you're interested in
7 studying.
8 So we used an antibody against H3K9,
9 acetylated H3K9, and then you separate your DNA,
10 amplify it, or put it on a ChIP or sequence it, or do
11 something. And you'll find out how histone that's
12 been acetylated is near the promoter region of your
13 gene.
14 So that's our basic ChIP analysis. And when
15 we did that, we found that both for CXCR2 and CXCL1
16 or KC, we had this enhanced level of acetylated
17 histone protein near the promoter regions of those
18 two genes, which helped to complete our
19 understanding.
20 By the way, if you do intrathecally inject a
21 little CXCR2 antagonist, you can reverse mechanical,
22 not thermal, sensitization in this particular model.

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1 So that worked out as well.
2 Now I'm going to change direction just a
3 little bit. And here I'm going to go back to the
4 observation that this chemokine signaling pathway,
5 epigenetically regulated, is seen not only in central
6 nervous system tissue, but in the periphery, it had
7 been looked at as well. And not only has it been
8 looked at in the periphery, but part of our
9 laboratory's interest happens to be in how opioids
10 might change chemokine signaling peripherally.
11 This was just happenstance, but we've had a
12 long interest in why patients who have had chronic
13 treatment with opioids seem to be so difficult to
14 manage postoperatively. And by the same token,
15 patients who have additional injuries who are
16 chronically managed with opiates tend to be difficult
17 as well.
18 So they have higher pain scores. They have
19 much higher opioid requirements. They have more
20 frequent side effects probably because you're pumping
21 more opiate into them trying to control the pain
22 postoperatively. And where it's been looked at,

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1 functional outcomes in these patients are also poor.
2 It is a problem area for clinical medicine.
3 Once we can model some of this, we can
4 certainly show greater sensitization, longer
5 duration. And some people in the audience have been
6 involved for years in doing just those experiments.
7 Again, coincidentally, we had several years
8 ago looked at what opiates did to peripheral levels
9 of cytokines and chemokines. One of those chemokines
10 we had included in the panels happened to be CXCL1,
11 which was known as KC at the time. And, in fact, in
12 the morphine-treated animals after incision, there
13 was more of it. We invoked that as an explanation
14 for why the animals had more pain-related behaviors.
15 So we're going to return. It was a
16 homecoming to that particular system. We used the
17 model that's displayed here. And basically what that
18 shows is if you make an incision in an animal here
19 and measure mechanical sensitization -- why you find
20 it -- and it gets better over the course of days. If
21 you give an animal morphine for 4 or 5 days, it too
22 becomes sensitive to noxious stimuli. If you stop

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

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

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

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

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

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

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

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

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

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

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

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

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

3 What we did was we studied their expression
4 in spinal cord tissue. We treated the animals for
5 4 days with morphine with or without the deacetylase
6 inhibitor SAHA. And then we waited a week, which is
7 really that period over which most animals recover if
8 they've just had morphine. But we asked for which
9 genes is there still an elevation if you block
10 deacetylation. That was our clue that there might be
11 something epigenetic going on.

12 It was really here for BDNF and prodynorphin
13 that we had the greatest evidence that there was this
14 persistent effect of the blockade of histone
15 deacetylation, and maybe NR2B as well. But we really
16 pursued BDNF and prodynorphin.

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

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

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

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

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

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1 together to make a pain problem a lot worse. And one
2 set of factors that does prolong postoperative pain
3 recovery, and is a clinical problem, is the use of an
4 opiate long-term plus having an injury like an
5 incision if you have an operation.
6 So here we're going to measure BDNF and
7 prodynorphin. This is the day after we cease
8 administering morphine. We find a little more
9 message for BDNF and a little more message for
10 prodynorphin. If we make incisions and look at those
11 the day after, yes, there's a little more BDNF
12 message, a little more prodynorphin, yes. And you'll
13 measure the protein, and it's up, too.
14 But if we do both things, we have a sort of
15 additive effect of doing both on the level of
16 message. And, in fact, I think the key part here is
17 that that additivity is seen in our CHIP assay. So
18 if you put drug plus injury together and you have
19 greater activation of that acetylation of the process
20 that's controlling the expression of those genes, I
21 think that is what we found most interesting.
22 So to end with a model, this is, of course, a

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1 very oversimplified cartoon, but it sort of is
2 driving conceptually some of what we'd like to do
3 experimentally. And what it represents here
4 fundamentally is lots of processes coming together to
5 converge on epigenetic mechanisms then ultimately
6 controlling pain sensitization.
7 A few of the key players are with additional
8 data that I've not shown you this morning, there's
9 classes of nociceptive neurotransmitters.
10 Neuropeptides are displayed here. Excitatory amino
11 acids do the same thing in our neuro cultures. The
12 presence of drugs can do this. Descending
13 facilitating or inhibitory fibers might drive this as
14 well.
15 But ultimately, we suppose those things might
16 converse on some set of epigenetic mechanisms. Here
17 I have drawn in the phosphorylation of CREB, which is
18 one plausible mechanism, but it could be many others
19 that then control the expression of a finite group of
20 genes in a specific group of cells controlling
21 nociceptive transmission.
22 So I'm going to end on that model slide, and

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1 then I guess invite any questions.
2 (Applause.)
3 DR. CARR: Hi. Dan Carr, Tufts University.
4 I very much enjoyed your presentation.
5 Very often there are claims made for dietary
6 supplements like the red wine, resveratrol and
7 curcumin. Would you comment on the doses that were
8 given understanding there are species difference --
9 DR. CLARK: Large doses of red wine.
10 (Laughter.)
11 DR. CARR: In your experiments, understanding
12 it may not be generalizable across species, are the
13 doses remotely akin to those that can be achieved
14 with dietary intake of turmeric?
15 DR. CLARK: This is a good question because
16 this is actually where some of these agents come
17 from. I think the dietary supplement we've used here
18 is curcumin. And it is very difficult to answer this
19 specific question, but I'll tell you what the
20 literature -- it's poorly absorbed.
21 It has been used in many clinical trials as a
22 drug in an attempt to better control cancers. The

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1 presumed -- I don't think people really knew what the
2 mechanism was, but one that was written about in the
3 discussion sections is, in fact, the regulation of
4 histone acetyltransferase.
5 They have been used in doses from milligrams
6 per day to tens of grams per day with serum levels
7 often documented to be below those, which probably
8 interact with the particular enzyme systems that they
9 would purport to control.
10 So I can't tell you that the doses we achieve
11 in the animals are things that we can in a human. I
12 think here, rather, we're just using it strictly as
13 probe compounds, and I really don't advocate people
14 go out and use a lot of curcumin around the time of
15 an injury, even though there is one very interesting
16 paper which shows people might recover a little
17 faster; not a terribly good study but intriguing.
18 DR. MAIXNER: Laura.
19 DR. STONE: The double whammy of being on
20 chronic opioids and then having surgery, that's the
21 population of patients with chronic back pain that
22 have tried all the drugs, are on chronic opioids, and

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

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

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

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

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1 2012, where they estimated that AstraZeneca was
2 spending about \$12 billion a year for each product
3 they launched. That is just unsustainable.
4 Frankly, many new medicines are also becoming
5 unaffordable. So you're all aware the Vertex
6 example, the cystic fibrosis drug, that they're
7 charging, what, \$300,000 a year. That's \$800 a day.
8 Who can afford that?
9 With an aging society, of course, we
10 desperately, desperately need more new medicines, and
11 also, diseases of modern living; I'm thinking of
12 diabetes, obesity, et cetera. The thing that
13 continually worries me is that in biomedical
14 research, in both academia and in industry, there is
15 massive duplication.
16 The tragedy is that most of our ideas, most
17 of the targets, most of the assets that we work on
18 when we take them into phase 2, they fail. This is a
19 horrendous waste of money.
20 The problem, the first one, target selection
21 is difficult. We've got hundreds of targets. We
22 could sit here and come up with a whole list of

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1 targets. Actually picking which one to invest in is
2 the problem. We're not good at it, even today in
3 2014.
4 The fact is complex diseases are all caused
5 not by a single gene, but they're polygenic. They're
6 caused by multiple gene defects. Complex diseases
7 are immensely heterogeneous. We've heard today about
8 some of these epigenetic environmental influences,
9 but there are sex differences, age differences,
10 chronicity, stress effects, drug exposures, early
11 life experiences, injury, et cetera. The whole body,
12 the nervous system and the immune system in
13 particular, are incredibly plastic.
14 What we know in the clinic is that dirty
15 molecules or combinations of existing drugs are
16 usually more effective. So I think one of the
17 challenges we've had in the past two decades is we've
18 been seduced by single molecular targets.
19 So my hypothesis is that many of these
20 epigenetic proteins that Steve touched on, I believe
21 they may represent single early stage mediators of
22 complex diseases. So by affecting one of these

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1 proteins, I believe we can actually target many of
2 these polygenic diseases, if you like, and actually
3 try and deliver something that's more optimally
4 effective.
5 The second problem is that in academia,
6 there's a lot of expertise and infrastructure and
7 resources, but academia lacks a lot of tools.
8 Academia lacks high quality, novel inhibitors for
9 novel proteins. So to dissect biology, we need those
10 tools.
11 Of course, PhRMA is brilliant is at medicinal
12 chemistry. They've got the resources, and they've
13 got the strength. And they're good at generating the
14 tools, but most of the tools sit inside PhRMA.
15 I mentioned that there's massive duplication
16 in biomedical research and there's too many targets
17 to choose from. So what we've chosen to do is we've
18 created a public/private partnership to generate
19 novel tools, inhibitors, and make them freely
20 available in an effort to crowd-source science, to
21 use the whole of global academia.
22 So this is what we do. We only work on new

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1 human proteins. We work on proteins that most other
2 people are not working on because we think this is
3 where the new drugs are going to come from. And for
4 those proteins, we generate what we call target
5 prioritization tools. We generate the human protein.
6 We generate assays for that protein. We generate
7 small molecule inhibitors for that protein. We
8 generate antibodies, and we generate the
9 three-dimensional X-ray structure.
10 I should say that generating each of these
11 inhibitors, these are incredibly high quality
12 molecules, better than molecules that have often been
13 generated at that stage within PhRMA. They're
14 costing us in the region of two and a half to
15 \$3 million each to generate.
16 But the thing is, all of these reagents, we
17 make them freely available. We give them away to
18 anybody in academia, anybody in biotech, and anybody
19 in PhRMA because we believe that's the best thing we
20 can do to facilitate science, and therefore
21 facilitate drug discovery.
22 As a consequence, we've come a hub for

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

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

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

22 We work on families of proteins. These are

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

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

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

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

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

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

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

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

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

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

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

22 Now, when we generated this inhibitor, we

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1 thought, okay, now what are we going to do with it.
2 And there was some literature that said that this
3 protein, BRD4, if it exists with another protein
4 effusion and not BRD fusion, then those patients
5 develop a very rare cancer called NUT midline
6 carcinoma. It's so rare many oncologists can't even
7 diagnose it.
8 We worked with Jay Bradner at Harvard. He
9 generated this data. He is the world expert in NUT
10 midline carcinoma. He has access to patient-derived
11 cells.
12 So what he did was a simple proliferation
13 assay and put the inhibitor on. So this is the
14 compound, and these are two patient-derived cells.
15 And you can see the compound reduces proliferation.
16 The compound reduces proliferation.
17 He also then, using annexin V and propidium
18 iodide, showed that it increases apoptosis. So for
19 example, this is in vehicle. Twenty-four hours after
20 the compound, it's gone up, and 48 hours later, it's
21 gone up even further. So apoptosis goes up.
22 Proliferation goes down.

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1 Then he put it into a xenograph model. So he
2 took these patient-derived cells, put them into a
3 mouse. The tumor volume got bigger and bigger over
4 days. This is the vehicle. This is the compound.
5 When we got this data, Jay wanted to take
6 this molecule into patients because there is nothing
7 out there that treats this site of cancer. Anybody
8 diagnosed with NUT midline carcinoma, they're dead
9 within three to six months.
10 Since then, we've given that molecule to more
11 than 400 labs across the world. They've now shown
12 that that molecule works in a whole range of other
13 cancers. There was also a paper in 2012 that the
14 compound could be a target for male contraception.
15 Last year, there was a paper in Cell that the
16 compound reduces cardiac hypertrophy. And then GSK
17 generated their own molecule and showed that it
18 prevented and inhibited LPS-induced endotoxic shock,
19 so septic shock. If you give the compound before or
20 afterwards, it stops the animals dying. This is the
21 vehicle treated. They're all dead in 40 hours, and
22 the compound stops it dying.

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1 I deliberately share this data because it
2 illustrates we do not know how to do target
3 discovery. Here's a new target, new molecule. I've
4 shown you data in cancer. I've shown you data as a
5 male contraceptive. I've shown you data in cardiac
6 hypertrophy. And here's the data in septic shock.
7 And this is the challenge that industry has when they
8 have these new targets, which disease area do they
9 take it forward?
10 Well, since we generated that molecule, let's
11 see what's happened. We've given that molecule to
12 more than 400 labs all over the world and many
13 companies. And, of course, I pay nothing for that.
14 GSK couldn't afford to do it. Pfizer couldn't afford
15 to do it.
16 We published that paper in Nature in December
17 2010. It's been cited more than 400 times since. As
18 a consequence of that one paper, there's now been in
19 three and a half years more than 200 papers on that
20 target, many of them using that molecule. This is
21 crowdsourcing science in a totally unprecedented way.
22 We've enabled proprietary efforts. Today,

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1 there are five companies with five molecules in the
2 clinic, again, unprecedented. And Jay Bradner
3 secured \$15 million of VC funding to set up a biotech
4 to take that target into the clinic. This has been
5 the impact of one probe freely available to the whole
6 of the biomedical community.
7 Let me give you another example, another
8 family of proteins, and we've heard the speakers talk
9 about it this morning, the demethylases. We worked
10 with GSK to generate an inhibitor of this demethylase
11 called JMJD3. You can see it's potent and it's
12 selective over these other demethylases.
13 We took that compound and put it onto human
14 macrophages, and it produced a dose-related reduction
15 in the release of TNF from human macrophages. Now,
16 anti-TNF, many of you're aware, are currently selling
17 \$27 billion a year for the treatment of RA and IBD.
18 Here's a new mechanism for reducing TNF production.
19 We put the same compound onto osteoclasts and
20 showed that it reduces bone resorption. We put it
21 onto human breast cancer cells and showed that it
22 increases apoptosis. Again, another new target, a

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1 new molecule, and I've shown you three different
2 indications: inflammation, bone, cancer. I would
3 argue there is nobody on the planet that can tell me
4 where that target is going to end up.
5 We've now generated 24 of these inhibitors.
6 I've just shown you details of two of them. We've
7 generated 24. We intend to generate about 10 more
8 per year in the coming years, and they're all freely
9 available to anybody who wants to use them. These
10 are the other epigenetic targets that we're working
11 on, and we're trying to optimize these molecules.
12 And I say again, each of these molecules is costing
13 us working closely with industry in the order of 2
14 and a half to \$3 million.
15 The third problem is target prioritization.
16 Now, I'm afraid I have little confidence in data in
17 cell lines. I think until some of the recent work
18 that Frank's been doing on animal models, I largely
19 believe animal models have not been predictive of
20 what happens in the clinic. I think often the
21 challenge we've got is that data generated in
22 different labs is often different and conflicting.

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1 So what we now intend to do is we're going to
2 set up a panel of primary human healthy and diseased
3 cells and tissues. We're going to focus on
4 inflammation initially, and then we're going to
5 systematically profile our normal inhibitors in these
6 cells.
7 So, for example, we can get T cells, B cells,
8 macrophages from healthy individuals and patients,
9 patients with MS, IBDs, RA, et cetera, et cetera. We
10 will compare our inhibitors in those assays, and this
11 will be a way of comparing healthy with disease,
12 disease with disease, target with target, and cell
13 type with cell type. I think this is going to be a
14 better way to discover new targets for inflammatory
15 diseases.
16 We've already started working with a company
17 called Discovery. This is data they've generated for
18 us for free. So they took a novel CREB inhibitor
19 that David talked about, David mentioned in his early
20 talk, CREBBP. This is a novel inhibitor of this
21 bromodomain protein. These are 12 different human
22 cells, different epithelial, fibroblast, smooth

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1 muscle. And basically what they've done is they've
2 profiled this inhibitor at four different
3 concentrations, as shown by these different colored
4 lines. And they've come to a conclusion that the
5 compound has antiproliferative immunomodulatory
6 effects matrix remodeling, and it also affects
7 angiogenesis.
8 This is just a quick way of trying to get a
9 steer of where these targets are likely to be useful,
10 and then we can probe the biology more deeply.
11 Here's another example, another inhibitor,
12 and you can see by comparison, that seemed to have
13 little effect. But there was again some suggestion
14 of these sort of effects.
15 As I said, we're now building a human immune
16 platform with all these different primary cells.
17 We'll do medium throughput screening using high
18 content readouts. It's going to be based in Oxford
19 but also at the Karolinska. We've just got a grant
20 from the Innovative Medicines Initiative to do this.
21 And our intention is once we've set this up, to apply
22 exactly the same to neurons derived from iPSCs from

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1 patients.
2 The fourth problem, there is massive
3 attrition in phase 2. Most novel targets fail in
4 patients. Most companies work on the same few
5 targets in parallel and in secret. Frankly, the way
6 we're doing drug discovery, we're wasting funds,
7 we're wasting people's careers, and we're needlessly
8 exposing patients to molecules destined for failure.
9 So what we intend to do, create a
10 public/private partnership to evaluate novel
11 inhibitors now in patients, sharing all the data and
12 all the reagents freely. If that's the cliff, that's
13 where at 9 times out of 10, we fail. Let's pool our
14 resources, do the experiment once. Let's do it well.
15 Let's share the data. Let's not do it 20 times in
16 parallel and in secret, and then not publish the
17 data.
18 This is what's happening in drug discovery.
19 We come up with a target. We run a screen. We get a
20 hit. We do lead optimization. We get a clinical
21 molecule. We make sure it's safe in animals, safe in
22 humans, and then efficacy in patients.

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1 This is the attrition at each of the stages.
2 Now, we could argue about the figures, but this
3 roughly my experience in GSK. We lose about half
4 here, 10 percent here, a third here, a third here.
5 But look at this.
6 Whenever we take a novel target -- and I'm
7 talking about novel targets, not me toos and new
8 formulations. We take a novel target into patients,
9 the failure rate is 9 in 10. And this frankly is
10 killing our industry. And of course, the tragedy is
11 this process takes five, six, seven years.
12 Now, that's a horrendous waste of money for
13 any one company, but when you think that 20 companies
14 are doing it in parallel and in secret, now when you
15 just add up how much money we're wasting, how many
16 careers we're wasting, but importantly, how many
17 patients we're needlessly exposing to molecules
18 destined for failure.
19 A target that we all know about and love,
20 TRPV1, this is just showing the patents that have
21 been taken out on that target in the past 15 years or
22 so color coded by company. It's not a complete list.

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1 It finishes at M. There's a lot more companies down
2 here.
3 Now, I can tell you my own story on this one.
4 I was working on this target in 1997. And then in
5 '99, 2000, Dave Julius and John Davies published
6 their knockout papers, and the whole industry jumped
7 on that target for pain.
8 In 2003 when I was in GSK, we came up with a
9 very potent selective molecule. We took it into the
10 clinic, and basically, there was no analgesia, but
11 there was an increase in body temperature. Now, we
12 didn't publish it, I'm embarrassed to say.
13 In 2006, there was still 60 companies working
14 on that target. And in 2010, there was still
15 academics, biotech, and PhRMA working on that target.
16 And then AstraZeneca published their clinical data,
17 no efficacy, increase in body temperature. Just
18 imagine in 13 years how much money we wasted on that
19 target and how many patients we exposed.
20 Now, this is color coded, the same target
21 TRPV1. Now the patents are color coded by
22 therapeutic area. Now, you can't see the list

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1 because I can hardly see it from here, but there's
2 allergy, Alzheimer's, asthma, COPD, headache, hearing
3 loss. I mean, the list just goes on, and it's not
4 even a complete list. The red is pain, and all the
5 others are different disease areas. Maybe this just
6 says we don't know how to do target discovery.
7 Now, I'm fortunate to be working with Tom
8 McCarthy, who's sitting at the back, who started this
9 company called Spinifex. And Andrew Rice is going to
10 give, no doubt, a brilliant presentation tomorrow on
11 some of our recent clinical data. But I wanted to
12 share two slides without stealing Andrew's thunder.
13 This is a clinical study that we completed
14 with this compound here in postherpetic neuralgia
15 patients dosed for four weeks. This is a placebo,
16 and this is the active compound. And you can see
17 clearly there's a significant difference down here.
18 Now, let me share with you the target. We
19 know angiotensin works at two receptors, AT1 and AT2.
20 If you block AT1, you get antihypertensives like
21 losartan. Tom's compound is a blocker of AT2.
22 Now, I know when we first got this data,

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1 there was many, many people in the pain area thinking
2 where the hell did this target come from. I can also
3 tell you if I was still inside GSK, there was no way
4 I would have been allowed to take an AT2 antagonist
5 into the clinic for pain because everybody thinks,
6 oh, it's cardiovascular, blood pressure. It's
7 nothing to do with neuroscience and pain. It's worth
8 bearing that in mind.
9 So what are we now going to do? We're now
10 going to take some new targets all the way through to
11 what we call proof of clinical mechanism, so
12 phase 2A, experimental medicine study.
13 In the process, we will generate these
14 reagents, shown here in blue, and we'll make them
15 freely available to anybody who wants them. We're
16 going to generate these publications shown here in
17 red. Those reagents in blue and the publications in
18 red, they will facilitate more collaboration, more
19 leverage funds, and more of these proof of clinical
20 mechanism studies.
21 So I may decide to take Target X into, let's
22 say, schizophrenia. But if Frank Porreca says to me,

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1 Chas, I think Target X is going to work in
2 osteoarthritis, we'll say, Frank, here's the
3 molecule. You go away and get your own funds and do
4 that clinical study. At least it saves Frank from
5 generating that molecule.
6 The patient groups we've talked to, they've
7 said that they will help us recruit patients, and
8 they'll minimize the payment. So potentially, the
9 studies could be cheaper and faster.
10 We've talked to many of our friends and the
11 regulators. They've said because this is a knowledge
12 creation endeavor, this isn't about me trying to make
13 money. This isn't about GSK trying to sell a drug,
14 et cetera. This is a knowledge creation endeavor.
15 They will help us design new clinical studies, help
16 validate new biomarkers, and help pave the path to
17 new targets, a totally unprecedented way of working
18 with the regulators.
19 Where have we got to? We've started the
20 project in cancer with Cancer Research U.K., the
21 world's biggest cancer charity. And on the 5th of
22 February this year, and we're sorting this out,

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1 Takeda has agreed to fund a new target for
2 Alzheimer's. Now, let me just repeat that, a new
3 target for Alzheimer's.
4 They want us to pick the target, which is
5 what we're doing now. They will then fund the high
6 throughput screen, the lead optimization, the
7 toxicology, the phase 1 and the phase 2A study
8 completely in the open. We're working with the
9 Canadian government, the CIHR, Canadian Institute for
10 Health Research. They want us to do this in
11 neuropsychiatry, and they're willing to fund \$30
12 million into this effort.
13 So this is what we've been doing. We've been
14 working on new targets. We've been generating open
15 high quality tools. We've been crowdsourcing
16 science. The green is what we've been doing.
17 What we've now started to do is we want to
18 profile these tools in human disease cells and start
19 generating open clinical candidates. And then
20 ultimately, we want to do open phase 2A studies.
21 This is what we intend to.
22 I think this should be the precompetitive

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1 boundary. Once we've worked out that 1 in 10 target
2 that's likely to do something, that industry can
3 generate proprietary assets. At least they know
4 they're working on a clinically validated target, a
5 de-risk target, not another TRPV1 or another NK1.
6 So summary. Target validation occurs in
7 patients. We have to get to phase 2A as quickly as
8 possible. Pioneer drug discovery, it's too high
9 risk, too expensive, and it takes too long. There's
10 too much duplicative activity on targets destined for
11 failure. We have to pool public and private
12 resources, access the best academics, work with
13 patient groups and regulators to de-risk pioneer
14 targets precompetitively. Industry can then generate
15 proprietary molecules for such targets.
16 I believe I think it is worth taking some of
17 our novel epigenetic probes and profiling them in
18 pain relevant cellular assays, whatever they may be.
19 Some of our molecules have got good PK in animals,
20 and it may be worth evaluating some of those in some
21 of the assays that people like Frank are developing.
22 And I think it's worth considering building a

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1 public/private partnership to take a novel target all
2 the way into phase 2A.
3 Ladies and gentlemen, we're doing this in the
4 interest of patients. Thank you very much.
5 (Applause.)
6 DR. MAIXNER: Questions?
7 DR. BASBAUM: Just going to play a little
8 devil's advocate. I won't quote the individual, but
9 it was somebody who we all know. And I remember
10 having lunch with this individual who told me that he
11 was incredibly successful in industry. Now, I happen
12 to know that he worked very high up at a company, and
13 when he was there, never got a compound into the
14 clinic that was successful. But he was convinced
15 that he was incredibly successful. Why? Because he
16 had brought 20 compounds into phase 3. And what he
17 was basically saying, my interpretation, is the
18 pipeline is what drove his market.
19 So success in that case meant that the stock
20 was doing incredibly well. So I wonder whether the
21 endpoint desire of PhRMA is not necessarily aligned
22 with the endpoint desire that we really want. That's

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1 a little bit -- it's an unpleasant perspective on the
2 industry, but are there -- somebody wants to make
3 money.
4 DR. BOUNTRA: Allan, of course, you're always
5 right. And the challenge we've got in this game is
6 that most of the targets, the new targets, that we
7 work on, we take them into phase 2, and 9 times out
8 of 10, they fail. And then the 1 in 10 that we think
9 works in phase 2, we take them into phase 3, and 6
10 times out of 10, they fail. They don't replicate in
11 phase 3. And that's, of course, where the company
12 spends an absolute fortune.
13 Then, of course, there have been many
14 examples where we've taken stuff to the market and
15 we've ended up pulling it because of side effects,
16 et cetera.
17 This is just such a difficult game. I
18 believe that industry has got some phenomenal
19 strengths. They've got strengths in areas that
20 require scale and infrastructure, so high throughput
21 screening, lead optimization, the tox, the regulated,
22 the really big clinical studies.

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1 But in academia, there are many things that
2 are easier to do. I didn't say we are better. I
3 said it's easier to do. In academia, it's easier to
4 work with clinicians. It's easier to access patient
5 material. It's easier to work with patient groups.
6 It's easier to access patient databases.
7 So what we're trying to do is to bring
8 together the strengths of both, but we're trying to
9 underpin it with public funding. We're trying to
10 bring in the regulators. We're trying to bring in
11 some of the patient organizations, et cetera,
12 et cetera, because frankly, this is the only way
13 we're going to do it.
14 I shared an example at Frank's meeting last
15 week in the Alzheimer's space, and I'll share it
16 again. In Alzheimer's, there's one target we've been
17 working on for 31 years, amyloid. The idea is this
18 protein accumulates in the brain. As a consequence,
19 neurons die. You get dementia, cognitive decline,
20 et cetera.
21 Thirty-one years, we've been working on that
22 target. If you added how much money we've spent in

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1 academia, in biotech, and in PhRMA, the estimate is
2 more than \$30 billion. After 31 years and
3 \$30 billion, we still don't know the answer.
4 Everybody is saying which form of amyloid, where in
5 the brain do you need to lower it, how much do you
6 need to lower it by, and if you do lower it, is it
7 going to have any effect, and when do you lower it?
8 We can't carry on like this. Alzheimer's in
9 one disease -- FYI, in the past decade, there's been
10 12 failed phase 3 clinical studies in Alzheimer's.
11 And many of those studies cost several hundred
12 million dollars. One of the Lilly studies cost
13 \$750 million. And this is one disease, if we don't
14 come up with a treatment in the next 20 years, it's
15 going to financially cripple many societies.
16 DR. BACKONJA: Misha Backonja from Salt Lake
17 City. You really make an excellent case for a way to
18 look at this problem in new ways. But the onus is
19 really on phase 1 and phase 2 trials. Spending the
20 past couple years in that arena, I realize that that
21 phase is really way beyond the times. There's so
22 much data that's not collected. It's not paid

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1 attention to.
2 So really to follow your model, the whole
3 idea of phase 1 and phase 2 has to be reconsidered
4 and redesigned and revised because, as I said, for
5 this to be a source of information and to help
6 facilitate translation, the data really needs to be
7 captured rather than kind of shunted aside, because
8 it's all down to this kind of mad rush to cross that
9 as fast as you can, really missing the opportunities.
10 DR. BOUNTRA: And I totally agree. Many of
11 my sort of clinical academic friends, they're sitting
12 on new ways of stratifying patients or new
13 biomarkers, but they need to test them. And they
14 need a molecule to test them, and they're willing to
15 do this. All they care about is just testing their
16 hypotheses. They're not worried about making money,
17 et cetera. So I think it's a beautiful intersection,
18 I agree.
19 Thank you, Bill. Thank you very much.
20 (Applause.)
21 DR. MAIXNER: Thank you, Chas.
22 Our next speaker is Dr. Jordana Bell from

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1 Kings College, and she will speak to us today on DNA
2 methylation studies in twins.
3 She is a senior lecturer in the Department of
4 Twin Studies. Maybe it's totally unique to Kings. I
5 don't know if there are other departments across the
6 world, but an amazing opportunity to dig deeply into
7 genetic factors by having a whole unit that focuses
8 on twin studies.
9 So, Jordana, we're very interested to hear
10 what you have to say.
11 Presentation – Jordana Bell
12 DR. BELL: Great. I'd like to thank the
13 organizers for inviting me to speak here.
14 So I'm coming to this from a slightly
15 different angle. My focus is very much DNA
16 methylation and other epigenetic processes in twins,
17 and one of the studies that we did was to apply these
18 methods to pain.
19 So first, disclosure. The bulk of the work
20 that I'm going to present today has recently been
21 published in this study. And on the far side are the
22 funding bodies that contributed to this, so Pfizer in

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1 part funded some of this work. In particular, Pfizer
2 paid for methylation sequencing in a subset of the
3 individuals that went into the final paper.
4 So I'm interested in looking at DNA
5 methylation variation in human populations and
6 looking at this from two perspectives. On the one
7 hand side, we have drivers of methylation variability
8 itself, and on the other side, we have potential
9 consequences of that variation to complex phenotypes.
10 So first, what potentially causes variation
11 in methylation? So if we consider the DNA
12 methylation landscape across the genome, we know that
13 we can identify very different subsets of regions.
14 So one subset of regions, which are so far a
15 relatively small subset of regions, we know that
16 genes or changes in DNA sequences strongly associates
17 with changes in DNA methylation profiles. So these
18 particular regions show very heritable patterns of
19 DNA methylation. And particular variants usually
20 very close to the methylation site itself, these
21 genetic variants strongly drive methylation
22 variability. So this is one set of regions.

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1 Another set of regions, we know that
2 environmental exposure drives variability in
3 methylation. And this is currently a very much hot
4 topic in research in many groups internationally, and
5 the type of factors that people are looking at
6 include diet, smoking, alcohol, chemical pollutants,
7 and many others.
8 In my opinion, the strongest evidence that
9 there is to date is for tobacco smoking. So I think
10 that 20 regions genome-wide have been identified
11 where methylation profiles in current smokers are
12 very different from those in nonsmokers. And
13 interestingly, ex-smokers show an attenuation, so
14 they fall somewhere in between. And some studies
15 show that it depends on when exactly they quit
16 smoking.
17 Another interesting link to this is that some
18 of these changes you also see in newborns from
19 mothers who smoke during pregnancy. So that's a
20 second set of regions where environmental exposure
21 leads to methylation variability.
22 Then there's a third set of regions that

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1 change presumably stochastically over time, and
2 research is ongoing in trying to exactly characterize
3 these changes.
4 Of course, these are the interesting regions.
5 There's also a whole other set of regions that don't
6 change over time, but we predominantly focus on the
7 variable regions.
8 The other aspect is how does methylation link
9 to complex phenotypes. So one simplistic hypothesis
10 here would be to say that methylation regulates gene
11 expression. So presumably especially in promoter
12 regions, changes in methylation would drive changes
13 in gene expression, which would lead to a complex
14 phenotype, but this is a little too simplistic.
15 So many of the changes that have been
16 identified in disease fall not just in promoter
17 regions but in enhancers and intergenic regions, and
18 gene body regions, which presumably also link to gene
19 expression regulation. But it's also very much
20 possible that a complex disease would change the
21 cellular environment, which would then drive changes
22 in methylation. So in that respect, methylation

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1 would be a marker of disease rather than a cause of
2 disease.
3 So I'm talking about twins. How do twins fit
4 into this? So one way would be to think of using
5 twin studies to understand more about the causes of
6 methylation variability, so applying the classical
7 twin study. So here methylation would be the
8 phenotype, and you would compare methylation profiles
9 in dizygotic or dizygotic twins who on average share
10 50 percent of their genetic variants to monozygotic
11 twins, who are genetically identical, to establish
12 what proportion of methylation variation is
13 attributed to genetic factors versus environment.
14 I'm not really going to talk about this
15 today. What I'm going to talk about is discordant
16 genetically identical twins.
17 So in this aspect, again, you can use twins
18 who are discordant for environment exposures such as
19 smoking to identify methylation markers of
20 environment exposure. But the focus of my talk will
21 be on disease discordant monozygotic twins. So one
22 potential mechanism that we're seeking to identify

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1 here is environmental exposure driving changes in
2 methylation, which then translates to changes in our
3 complex disease.
4 So discordant monozygotic twins, you would
5 think are very rare because monozygotic twins
6 themselves are relatively rare events. So twinning,
7 about one in 250 individuals has an identical twin.
8 But actually, if you look at some rates, it turns out
9 that concordance rates for some typical common
10 diseases are not as high as you might expect.
11 So here's some estimates from published work
12 as well as our own cohort. In fact, discordance for
13 some of these common traits ranges between 40 to
14 80 percent, so there's quite a lot of variability.
15 And you do identify discordant monozygotic twins.
16 So one could say that disease discordant
17 monozygotic twins is an ideal study design for
18 identifying non-genetic risk factors in disease
19 because compared to unrelated individuals, they are
20 controlled for the same germ line genetic variants.
21 Somatic variants are a different story.
22 They have the same or very similar in utero

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1 environment, and they're controlled for age, sex and
2 cohort effects. And it's the environment that really
3 varies, so similar early life but different adult
4 life environment.
5 So to date, people have used this design to
6 identify environmental risk factors in disease, for
7 example, alcohol and bone mineral density. And
8 currently, many studies are using this design to
9 identify molecular mechanisms and disease such as
10 somatic DNA mutations and epigenetic changes.
11 So I'm going to talk about the epigenetic
12 changes in a discordant twin design, but before I get
13 to that, just a few slides on doing an epigenome-wide
14 association study.
15 So many groups are currently performing
16 epigenome-wide association studies to identify DNA
17 methylation changes and disease. Typically most
18 would use a case control design using unrelated
19 individuals. There are also a bunch of groups using
20 the discordant MZ pair design. And I guess in
21 addition to the potential confounders that I
22 mentioned before, one potential benefit of comparing

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1 these different types of design is that in the
2 unrelated case control design, you might be able to
3 identify genetic changes that associate with the
4 phenotype that are mediated through DNA methylation
5 variation, which would be of interest to dissecting
6 the molecular mechanism of disease, while with the
7 discordant twin design as discussed, presumably
8 you're looking for environmentally driven changes.
9 But both really cannot tell you -- they
10 cannot establish whether this is a cause or a
11 consequence completely. So ideally, what you really
12 want is to have some longitudinal samples where you
13 can look at DNA methylation over time prior to
14 disease onset and post disease onset.
15 Here's some of the issues that one needs to
16 keep in mind when conducting an EWAS study. So
17 clearly, EWAS study design and power and sample size,
18 so most of the DNA methylation changes link to a
19 complex disease that have been identified to date are
20 relatively modest. So clearly, you need large
21 samples.
22 Are you in the right cell and tissue for the

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

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

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

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

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

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

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

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

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1 that temperature is the phenotype.

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

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

14 So briefly, we took this approach, and at the
15 end of the day using a number of normalization
16 techniques for strand bias and so on, we ended up
17 with approximately 5 million overlapping 1kb regions
18 across the genome. So at each one of these regions,
19 we looked at the DNA methylation profiles across the
20 50 twins.

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

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

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

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

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

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1 twins are highly concordant for ASM skew or which
2 allele is methylated.
3 third, this relates to the assays of DNA
4 methylation. So we used MeDIP sequencing, which has
5 very good coverage, but it's not very sensitive
6 because you can only go down to 200 base pairs. So
7 you cannot get the single base pair prediction of
8 methylation. So when we compare our data to a
9 different array which is single base pair array but
10 has relatively poor coverage, then we see good
11 correlation across the two.
12 So then moving on to comparing methylation to
13 pain, when we compare methylation to pain across the
14 entire data set of individuals, here are the results.
15 So this plot shows the Q-Q plot of the observed
16 versus expected data. In black are the observed
17 results, and the shaded area in gray is what we
18 expect to see under the null hypothesis that pain
19 does not associate with methylation. And this was
20 done by permutation.
21 So relatively modest enrichment of signal
22 basically. And overall, there was one genome-wide

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1 significant region. And this region did not fall in
2 a gene.
3 So as I said, once we did this, we know that
4 the majority or a large proportion of the genome is
5 not variable in terms of DNA methylation patterns.
6 So what we did then is to only look at the proportion
7 of the genome that falls in interesting functional
8 genomic categories. And specifically, we looked at
9 CpG islands. We looked at the regions right at the
10 borders of CpG islands, which are called CpG island
11 shores that others have shown to be functionally
12 interesting, promoters and other regions. And what
13 we saw was a weak enrichment when we look
14 particularly in CpG island shores.
15 So then we went on, and we looked at what
16 hits are we getting specifically, just looking in the
17 top 100 ranked hits across all different analyses.
18 And this is what we get.
19 So genome-wide, looking at 5 million tests,
20 when we examined the top 100 hits, we saw some
21 interesting genes in that list. And importantly,
22 TRPA1 was ranked top 44th. When we then look at CpG

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1 island shores, TRPA1 is now ranked top fourth, and we
2 also see V1 and V3 in that list of top 100 genes.
3 This is only 50,000 tests genome-wide. And then when
4 we restrict it to CpG islands, we see some more
5 interesting genes.
6 If you look at gene ontology analysis of
7 these sets of genes, then you do come up with terms
8 that are relevant to pain.
9 So at this point, we stopped this pilot
10 study, and we decided that the results we were
11 getting were interesting. And we decided to spend
12 some more money and extend the study to a larger
13 study design.
14 So here's the final study design. So what
15 I've told you so far is the discovery stage in
16 monozygotic twins. We then went on and profiled
17 another 50 unrelated individuals where the HPST
18 scores were just representative of the cohort.
19 We did the same type of analysis that I
20 showed you so far. We then meta-analyzed the
21 findings, took the top hits from the meta-analysis,
22 and went on to validate them.

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1 So the validation included two analyses which
2 basically validated the methylation signal we were
3 getting using a different methylation assay based on
4 bisulfite conversions, so either bisulfite sequencing
5 or this Illumina 450k array that I mentioned. And we
6 also did a longitudinal follow-up where we took 33 of
7 the original twins, and we recalled them back in
8 clinic three years later, and we looked at their HPST
9 and methylation profiles.
10 So from the meta-analysis across the 100
11 combined individuals, what do we get? We were very
12 excited to see that the top region genome-wide was in
13 TRPA1. And in addition to TRPA1, there were
14 altogether nine regions that surpassed statistical
15 significance at a permutation based, false discovery
16 rate of 5 percent.
17 These nine regions actually fall in eight
18 unique regions. ST6GalNAc3 appears twice. It's a
19 contiguous DMR. So we thought that TRPA1 and
20 potentially some other genes in this list where quite
21 interesting. We focused on TRPA1.
22 I think many of you know TRPA1 much better

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

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

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

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

22 What we see in skin is, as expected, a

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

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

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

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

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

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

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

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

6 Where do the nine DMRs fall in this? So
7 nearly all of them -- I mean, it depends what you
8 call variability and what is not variability, but
9 most of them show a very stable pattern over time.

10 So we conclude that the top 9 results are relatively
11 stable over time.

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

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

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1 So what we did is for the top 9 regions, we
2 corrected the results because in a subset of
3 individuals, we had facts data from lymphocytes,
4 neutrophils, monocytes and eosinophils. And we saw
5 that generally the results were very similar within
6 one region, on chromosome 4, the top 6 region showed
7 an associated with lymphocyte count.
8 The other issue was okay, well, blood is
9 clearly perhaps not the best tissue for pain
10 sensitivity, and so we looked at data across
11 different tissues. And we had access to a study of
12 two female donors for whom we had blood and
13 methylation profiles from seven brain regions, and we
14 compared demethylation profiles across these
15 different regions. And we saw that five of the top
16 pain DMRs showed very similar patterns. But in
17 general, that data was a little bit difficult to
18 interpret because there's quite a lot of variability.
19 For example, cerebellum and brain was as different as
20 blood and brain.
21 So in conclusion, that study used a
22 discordant monozygotic twin design to assess the

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1 effect of methylation on pain. We identified some
2 differentially methylated regions that associated
3 with pain, and these validated using a different
4 technique. We combined the twin design with an
5 unrelated design to try and understand more about the
6 mechanisms driving the methylation variation and the
7 epigenetic associations.
8 I think in general, epigenetics can
9 complement and extend genetic studies to help us
10 understand molecular mechanisms in disease. And this
11 was a pilot study of a larger project, the EpiTwin
12 Project in which we're profiling methylation of 5,000
13 twins in blood using predominantly methylation
14 sequencing as well as array data.
15 Here are some of the traits that we're
16 looking at the moment. In red are the ones that
17 we've completed, and for each of these, we have
18 profiled methylation predominantly focusing on
19 disease in discordant monozygotic twins. We're
20 performing epigenome-wide association and exploring
21 the differentially methylated regions in disease.
22 Thank you.

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1 (Applause.)
2 DR. MAIXNER: Questions?
3 DR. PORRECA: A very, very interesting study.
4 I was thinking about the identifications of the
5 differences in methylation in the different genes
6 that you were finding and your link of those to pain.
7 And I guess this is -- I mean, what if the twins have
8 other changes going on that are still linked to that
9 particular gene, such as TRPA1, for example?
10 That is not necessarily -- that is identified
11 by the pain sensitivity, but it's actually not linked
12 to pain. I guess I'm not being very clear on that
13 question. But how do you actually link it to that
14 pain sensitivity I guess is what I'm trying to ask.
15 DR. BELL: So I guess another way of saying
16 that is you're saying is there a confounder that
17 is -- I mean, we tried to --
18 (Laughter.)
19 DR. BELL: We tried to control for potential
20 confounders as much as we could. And so age, BMI,
21 these are all female twins. Smoking status and so
22 on, we explored all of these. We also explored cell

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1 composition because we thought that might influence
2 the effects. And as much as we could, none of these
3 factors confound the finding. But of course, there
4 are many other confounders for which we simply don't
5 have data, especially environmental exposures is one.
6 And characterizing the twins very well is very
7 difficult to do that.
8 I mean, for TRPA1, it was quite interesting
9 that we not only see it in the twin sample but also
10 in an unrelated sample that was completely
11 independent. So that gives me confidence to say
12 there's likely something there. Of course, it could
13 be confounded, but until we have better environmental
14 data, I can't really answer that question.
15 DR. BOUNTRA: Jordana, could I ask Frank's
16 question in a slightly different way? Do you believe
17 that TRPA1 is more likely now to be a therapeutic
18 target?
19 DR. BELL: That's a difficult question. I
20 believe that there's a methylation change that
21 associates with a measure of pain. So I believe it
22 could be a marker of pain. I think our study on its

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1 own cannot at all say whether it's a potentially
2 causing pain or a consequence of pain. I think a
3 follow-up needs to be done with the proper experiment
4 in a model organism presumably to address causality.
5 DR. BASBAUM: This is again a follow-up to
6 Frank's attempts.
7 (Laughter.)
8 DR. BASBAUM: So A1 has been implicated in a
9 variety of respiratory conditions, asthma, for
10 example. Do you know anything about the status of
11 the twins with respect to asthma?
12 DR. BELL: We have phenotype data on that. I
13 haven't looked in the sample, but we could look at
14 TRPA1 on asthma. We have data on those. I haven't
15 looked at this.
16 DR. BASBAUM: Thanks.
17 DR. MAIXNER: Thank you very much.
18 (Applause.)
19 DR. BASBAUM: Try to be lighthearted around
20 lunchtime. So I get to introduce Bill. And I didn't
21 think I needed to do any reading to know about him
22 because I've known Bill for so long. But I did

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1 learn -- I mean, I know he got his PhD at University
2 of Iowa in pharmacology and his dental degree there,
3 and he went to NIH.
4 This the part I had to write down. He is the
5 Mary Lily Kenan Flagler Bingham Distinguished
6 Professor -- that is very impressive -- at the
7 University of North Carolina. And he is also
8 director of the neurosensory disorders group in the
9 Department of Endodontics.
10 A few years ago, he turned to genetics and
11 has done some spectacular work specifically
12 concerning pain, and we're going to hear about that
13 today.
14 Presentation -- William Maixner
15 DR. MAIXNER: Thank you, Allan.
16 I want to thank the organizers for the
17 opportunity to present some of our actually
18 historical and some ongoing work that we've been
19 doing with many collaborators across the country.
20 Actually, disclosure, I should disclose I'm a
21 cofounder and equity shareholder in a small biotech
22 company called Algnomics, which focuses on

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1 diagnostics and therapeutics.
2 So today what I'd like to do is really cover
3 two general areas, a heuristic model, which many in
4 this room have seen, but each time I look at it, I
5 see something different. It is a mosaic, a model
6 that is a mosaic, which can become a kaleidoscope as
7 one works through it. And then I'd like to show a
8 single example of a translational approach that our
9 problem is using to identify putative targets in the
10 pain area, and based on that identification, to go
11 through a series of translational steps, which we
12 feel are important in validating and potentially
13 bringing a drug to phase 2.
14 We call it the translational clock, which was
15 in the title, and we'll discuss and I'll present our
16 approach to the translational clock, which is
17 essentially a reverse translation going from human
18 genetic association studies, identification of
19 potential pathways, identifying key elements of those
20 pathways that are potentially druggable, and then to
21 show proof of concept or proof of principle using
22 that approach, with the desire of really identifying

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1 both novel targets and also trying to speed the
2 development and identification and validation of
3 target.
4 So first, let's start with the conceptual
5 model. Many have seen this before. This is a model
6 that was developed several years ago, published back
7 in 2006 by my colleague Dr. Luda Diatchenko, and many
8 of us in the field, myself, Roger Fillingim, several
9 were involved in putting this together.
10 It's really not a quantum leap. This is
11 really an attempt to begin to integrate several
12 features that we think are important in -- in fact,
13 not what we think, the field thinks is important in
14 contributing to the onset and maintenance of a
15 variety of complex persistent pain conditions. And
16 many of these features, we've already spoken about
17 this morning.
18 So there's much literature, much research
19 that shows that both psychological factors and
20 factors involved in pain transmission, pain
21 modulation, interact in a way that produces
22 temporally dependent, temporally dynamic changes in

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1 the signs and symptoms that a patient expresses in
2 the clinic.
3 So if we look at these black arrows
4 underlying the ovals of psychological distress and
5 pain amplification, there's several intermediate
6 phenotypes or endophenotypes, which can be assessed
7 in a given patient and in a population of patients
8 that expresses signs and symptoms that are collected
9 in the clinic.
10 These intermediate phenotypes are temporally
11 dependent, and they depend largely on environmental
12 exposure, the types of exposures that the individual
13 experiences during their lifetime. And several of
14 those exposures, at least the nature of those
15 exposures, are shown on the Y axis as various
16 variables, which are involved in inducing
17 methylation, histone changes, so physical trauma,
18 abuse, infection, psychological life stressors, and
19 chemical events such as exposure to cigarettes.
20 We in OPFERA just recently published that the
21 odds of being a chronic pain case versus a control
22 non-pain case is about 2.4 if you have a history of

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1 smoking at least 100 cigarettes in your lifetime. So
2 this goes well with some of the previous statements
3 made that exposure to certain chemicals can produce
4 long-term changes in the organism that not only is
5 detrimental in the short term but produces long-term
6 changes that put them at risk for developing a
7 variety of conditions, including chronic pain.
8 On the X axis is merely a schematic of
9 several genes, multiple genes. As noted this
10 morning, I think few of us believe that there is a
11 pain gene. There are many genes that contribute to
12 complex and common chronic pain conditions. And
13 polymorphisms in each of several genes contribute
14 differentially to produce this temporally dependent,
15 very dynamic changes in signs and symptoms and
16 susceptibility to a variety of chronic pain
17 conditions.
18 So we see that environmental exposures
19 through epigenetic mechanisms that we've spoken about
20 today can change the expression patterns of multiple
21 genes, each which have potentially small effects on
22 the intermediate phenotypes and which are temporally

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1 changing like the city lights that Laura spoke about
2 earlier.
3 One important feature to remember that we
4 haven't really gone in detail is that chronic pain
5 patients, patients with fibromyalgia, TMD,
6 vulvodynia, lower back pain, as well as individuals
7 who are going to experience postoperative pain,
8 they're not a homogenous population.
9 There's great heterogeneity in the
10 population, and one of the tricks that we have, as
11 Chas mentioned, is to somehow identify stratification
12 procedures that permit us to begin to differentiate
13 individuals into subgroups based on phenotypes, these
14 intermediate phenotypes, and then based on those
15 phenotypic clusters, the molecular profiles that
16 underlie those clusters.
17 Unfortunately, I won't have time to show you
18 some of the approaches that we've been taking in some
19 of our large cohort studies, but this is a very
20 important trend in the field and now has begun to
21 respect the pleiotropic manifestations that these
22 individuals express with and try to capture those

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1 manifestations by cluster analysis in particular; and
2 then to look at the specific molecular profiles,
3 looking for specific targets that may underlie these
4 specific clusters.
5 So I'd like to give an example of how we use
6 the translational research clock, which we recently
7 published in Nature Rheumatology. And again, this is
8 a reverse translational process where we start with
9 the genetic association studies, confirm
10 functionality using cellular systems, then move to
11 animal models to show that the target or the putative
12 target has biological relevance. And then if we have
13 an agent in hand, a proof of concept trial leading to
14 some validity of the target.
15 I will say that we've evolved this
16 translational clock several times. The example I'm
17 going to show relates to catecholamine-o-
18 methyltransferase and the identification of a beta 2,
19 beta 3 pathway, and a drug that's been developed in
20 this area. We have identified the EGFR pathways, a
21 very important pathway in many of our cohorts, and
22 have a paper under review in Cell now that I think

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1 will help demonstrate that using this reverse
2 methodology, the EGFR pathway, and various inhibitors
3 of that pathway may prove to be very potent
4 analgesics in both the chronic pain and acute pain.
5 We also have identified new splice variants
6 in the mu opioid receptor using this approach. And
7 then finally, the angiotensin 2 pathway, we observed
8 back in about 2006 and are still working on some
9 additional data sets related to angiotensin 2.
10 So there are many other examples, but those
11 are four. And I'll present in some detail the one
12 that is most mature in this translational experience.
13 So starting with the genetic association
14 studies, Luda and group identified in 2005 genetic
15 variants in the gene that codes for catecholamine-o-
16 methyltransferase as an important genetic variable
17 that's associated with human pain sensitivity and the
18 risk of developing a condition known as TMD.
19 Since this study, there have been literally a
20 couple of hundred studies now that have validated
21 COMT as an important enzyme in the pain system and
22 that it is polymorphisms in COMT, especially those

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1 polymorphisms which lead to a reduced expression of
2 COMT. And potentially high catecholamine burdens in
3 the organism lead to increased pain sensitivity and
4 greater likelihood of developing a variety of chronic
5 pain conditions.
6 So this is an example of one gene that has
7 pleiotropic effects across several of the domains I
8 showed in the heuristic model and may contribute to a
9 variety of pain states.
10 Now, downstream from COMT are obviously,
11 adrenergic receptors, which are sensitive to
12 catecholamines. And we've published that
13 polymorphisms in the beta 2 adrenergic receptor are
14 associated with both psychological profiles,
15 psychological distress, alternations in blood
16 pressure regulation, and increased risk of TMD as
17 well.
18 We have similar examples for the alpha-1A and
19 beta 3 adrenergic receptor as well. So starting with
20 COMT as, if you will, a hub enzyme which is
21 controlling catecholamines that influence a variety
22 of processes, which I'll review in a moment, we've

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1 been able to identify a putative target, potentially
2 the beta 2 adrenergic receptor or the beta adrenergic
3 pathway, which may be important in modifying and
4 modulating pain and risk for a variety of conditions.
5 I'll just note that in the genetic studies
6 that were conducted by Luda and group, we identified
7 three SNP combinations, three haplotypes, that were
8 associated with differential pain sensitivity in the
9 human population. One haplotype we called low pain
10 sensitive because it was associated with reduced pain
11 sensitivity, where we phenotyped across five
12 different measures, thermal, mechanical, ischemic
13 measures. And using a combined normalized Z score
14 associating the haplotype with specific measures of
15 pain sensitivity, we identified a low pain sensitive,
16 an average pain sensitive, and a high pain sensitive
17 grouping of haplotypes, which explained about
18 80 percent of the population that was examined in
19 this study.
20 So we had an initial association study
21 pointing towards the COMT pathway and potentially
22 beta 2 adrenergic pathway, but then we wanted to ask

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1 the question as to whether there's some functional
2 relationship between these polymorphisms, these
3 haplotypes, and a biological pathway that was
4 functional in nature and not merely an association.
5 So we published then shortly thereafter the
6 findings that alteration in transcript expression
7 associated with these three haplotypes led to
8 different levels of protein expression. So in
9 panel A is basically the COMT activity based on
10 whether cells were transfected with the LPS, APS, or
11 HPS haplotype. And you can see cells transfected
12 with the HPS haplotype showed very little enzymatic
13 activity.
14 In panel B, which is partially covered, if
15 you look at the transcript levels generated by these
16 three cell lines that were transfected, you see that
17 there was surprisingly no change in transcript level.
18 And then if you look at transcript survival time in
19 panel G, what you see is actually the HPS transcript,
20 which again codes for the lowest amount of protein,
21 had the greatest survival time in the group assay, a
22 paradox, low protein levels, transcript levels the

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1 same and survival time greatest for the transcript
2 that's actually producing the least amount of
3 protein.
4 We were able to go on and show, using a
5 variety of informatic tools and site-directed
6 mutagenesis procedures, that in fact what is
7 happening is that individuals who harbor the HPS
8 haplotype code for a transcript which has much
9 greater free energy for uncoiling so that translation
10 into protein is greatly impacted by the amount of
11 free energy that's required at the transcript level
12 to induce into produce translation.
13 In contrast, those harboring the LPS
14 haplotype, the amount of free energy for uncoiling
15 and translation is much less, so it represents a
16 relatively novel and an important mechanism by which
17 we can influence translation into protein that
18 doesn't involve promoter site.
19 Whether they're epigenetic phenomenon that
20 are involved in transcript production outside of
21 promoter site, I think is an important question that
22 may also influence some of these patterns that

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1 develop.
2 So in 2006, we demonstrated that we, in fact,
3 have a functional consequence of these polymorphisms,
4 and then that then begged the question as to whether
5 we could show some type of an effect in the whole
6 organism. In various types of in vivo models are we
7 able to show that when we suppress COMT activity, are
8 we able to create a human phenotype, which mimics the
9 human condition?
10 Andy Nackley, who at that time was a
11 postdoctoral fellow with Luda and myself, conducted a
12 series of studies where she showed that using
13 different COMT inhibitors in rodents, that she could,
14 in fact, induce a human phenotype associated with
15 increased thermal pain sensitivity, mechanical pain
16 sensitivity, changes in bowel function, changes in GI
17 function beyond bowel function, and changes in
18 locomotor activities suggestive of a very complex
19 phenotype that was being produced by this inhibition
20 of COMT activity.
21 When we tried to examine what types of
22 downstream effectors may be involved with this,

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1 looking at alpha, beta, dopaminergic antagonists, we
2 were able to demonstrate that the use of relatively
3 selective beta 2 and beta 3 receptor antagonists were
4 able to restore or rescue that phenotype in the rat.
5 So this just shows data on the upper left
6 panel on mechanical threshold following the
7 administration of a COMT inhibitor, in orange, the
8 mechanical allodynia that develops. And this can be
9 rescued -- the behavior can be rescued with a beta 2
10 selective and a relatively selective beta 3
11 antagonist given in combination. If we give them
12 individually, we restore about 50 percent of the
13 phenotype. And we see this for both mechanical
14 threshold and mechanical hyperalgesia.
15 The lower panel shows the effects on thermal
16 pain. We see a thermal hyperalgesia that is
17 expressed with the COMT inhibitor, and again, rescued
18 with the combined combination of a beta 2, beta 3.
19 Coincident with these behaviors, we also see
20 elevation in circulating cytokines, IL-6, TNF-alpha
21 and IL-1 beta, and those can be restored with beta 2,
22 beta 3 antagonist.

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1 So we have in a short period of time, in two
2 years, identified a putative target, something that
3 would block beta 2, beta 3 and that would, in fact,
4 potentially be useful target to assess in a clinical
5 trial.
6 So in 2010, we conducted a small clinical
7 trial on 40 individuals, and I'll show in just a
8 minute. And we showed that the nonselective beta
9 antagonist, propranolol, which blocks beta 1, beta 2
10 and beta 3 somewhat weakly, is able to reverse and to
11 ameliorate the signs and symptoms of TMD, a very
12 common chronic musculoskeletal pain condition.
13 In this study, 40 individuals were recruited
14 with chronic TMD. A two-period, double blind,
15 placebo controlled randomized crossover design was
16 used. And a very low dose of propranolol was used
17 for seven days, wash out, and then seven days with
18 either propranolol or placebo. So there was
19 randomization by treatment arm as well.
20 This data just shows the pain relieving
21 effects of propranolol versus placebo. In this
22 study, we used what we called the pain relief index,

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1 which was essentially the average pain at a given
2 epoch in time multiplied by the percentage of time
3 during the waking day that the individual experiences
4 pain.
5 So this is a way of integrating the severity
6 of pain over time. So it's an average pain
7 multiplied by percentage of time during the waking
8 day that the individual experiences pain. And we
9 find this to be a very sensitive index for pain
10 assessment and pain relief because it respects the
11 temporal characteristics of change in pain.
12 On the far left independent of genotype, you
13 see the pain relief index, which represents about a
14 50 percent reduction relative to their clinical pain
15 at baseline. And if we stratify these individuals
16 based on genotype, a COMT genotype, individuals who
17 had zero copies of the low pain sensitive
18 haplotype -- remember, this haplotype is coding for a
19 lot of enzyme, a lot of COMT. So we're really
20 chewing up catecholamines in these individuals, and
21 so they have very little, we think, catecholamine
22 burden.

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1 These individuals who have zero copies of LPS
2 have a lot of catecholamine burden because they have
3 very low levels of COMT. They're expressing high
4 catecholamines because they're not able to metabolize
5 it as well. And because of that, we believe
6 propranolol is more effective in this subpopulation.
7 Individuals who have two copies of LPS are
8 the population that can really chew up catecholamines
9 and are in essence free of the catecholamine burden,
10 and we believe should be less sensitive to the
11 effects of propranolol. And about two-thirds of the
12 population carry either zero or one copy of the LPS
13 haplotype.
14 So this really caused us a lot of thought as
15 we looked at these studies, which occurred in a
16 relatively short period of time, as to whether beta
17 adrenergic receptors could play a role in pain or
18 pain processing and represent potentially new drug
19 target. And there's considerable evidence in the
20 literature that beta adrenergic pathways play a role
21 in pain, involved especially the beta 2 receptor, is
22 associated with hyperalgesic responses.

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1 John Levine and coworkers and many others
2 have shown that the application of beta 2 receptor
3 agonists, both centrally and peripherally, can induce
4 hyperalgesic states involved in human models of
5 arthritis. High dose propranolol has been shown to
6 be effective in treating rheumatoid arthritis. A
7 number of inflammatory cytokines and proteins like
8 NGF are regulated by beta 2 receptors.
9 We know that many ROS products released in
10 the periphery and the CNS that are able to produce
11 pro-pain and pro-inflammatory states can be
12 diminished with beta adrenergic blockade.
13 We know that beta 2 receptors activate
14 microglia when agonists are present. Beta 2
15 receptors stimulate classic cascades involved in pain
16 pathways. And EGFR, which we are showing to be an
17 important pathway in pain, is also regulated by beta
18 adrenergic receptors. And finally, there's a
19 considerable work that beta adrenergic receptors
20 contribute, as David has shown, to opioid-induced
21 hyperalgesia, tolerance, and seem to be also involved
22 in opioid sparing in the postoperative environment.

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1 So there are a number of attractive features
2 about the beta adrenergic receptor and agonists that
3 would suggest that it's worth pursuing the
4 identification of new chemical entities. And I can
5 say that our program over a two-year period spent
6 considerable time trying to identify a new chemical
7 entity that would be superior to propranolol as a
8 model therapy, as an analgesic, and potentially as an
9 agent that is able to produce opioid sparing.
10 Our search resulted in a kissing cousin of
11 propranolol called bupranolol, and bupranolol has a
12 number of very unique features. If you look at its
13 ability to block beta 1, beta 2, beta 3 receptors and
14 HEK cells that are overexpressing a specific
15 receptor, you see in panel A the dose response to the
16 agonist isoproterenol.
17 Using the EC50 for each of these, we then
18 were able to show that the r-enantiomer of bupranolol
19 is able to produce a new dose-dependent suppression
20 of beta 1 activities. It also has very nice beta 2
21 receptor activities, antagonist activity, and is able
22 to block beta 3 receptors as well. And it has a

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1 number of pharmacodynamic properties that appear to
2 be superior to propranolol.
3 Though I don't show it, propranolol actually
4 is a fairly effective agonist of the beta 3 receptors
5 at low to moderate doses, and the r-enantiomer of
6 bupranolol fails to show that agonist behavior.
7 If we look at some of the pharmacodynamic
8 properties of these agents, S bupranolol and
9 R bupranolol versus propranolol, we find that the
10 r-enantiomer of bupranolol is able to produce nice
11 dose-dependent reductions in the formalin tests.
12 Both the early phase and late phase, in inflammatory
13 models, caragenin model, the r-enantiomer of
14 bupranolol is able to rescue the inflammatory state
15 induced by caragenin.
16 This just shows the relative potency of the
17 r-enantiomer, s-enantiomer of bupranolol versus
18 propranolol. And R appears to have a nice profile
19 relative to propranolol. Neuropathic pain, the same
20 story, that the r-enantiomer seems quite able to
21 reverse a neuropathic pain state as well.
22 If we look at sedation using rotarod ataxia,

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1 you see that both enantiomers of bupranolol are
2 relatively free of ataxia at 60 milligrams while
3 propranolol is really knocking them off the rod. And
4 if you look at the therapeutic index, you see that
5 both the R and S show superiority to propranolol for
6 therapeutic index as related to lethality in mice.
7 These behavioral data were collected by Loren
8 Martin and Jeff Mogil at McGill.
9 So finally, what I'd like to do is just make
10 a few comments about future direction and the
11 translational research clock. I think this is one
12 example whereby one can reverse engineer, if you
13 will, from genetic variables back to biological
14 plausibility and identify either new targets that can
15 be druggable by existing agents that can be pulled
16 off the shelf, repurposed, or the creation through
17 synthesis of new chemical moieties.
18 I think this is one of several future
19 directions whereby target identification and I think
20 facilitation to proof of concept in a phase 2A is
21 expedited in a substantial manner. So I think this
22 is one of many ways to the future, and I think the

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1 application and the collection of very large cohorts
2 and stratification procedures will help in the
3 identification of large subpopulations in the pain
4 field that will benefit from this type of approach in
5 identifying putative targets that can rapidly be
6 validated in animal models and then moving forward
7 clinically.
8 Finally, I'd like to thank my many, many
9 colleagues who've contributed over the last several
10 years to both conceptual framework in the conduct of
11 these studies and to really the thousands of patients
12 who have contributed to our cohort studies over the
13 years, who've made some of these initial thoughts
14 possible. And I thank you for your attention.
15 (Applause.)
16 DR. CLARK: Thank you very much, Bill. I've
17 admired your work for many years.
18 You mentioned here and key on variants of the
19 catechol methyltransferase gene, and then the other
20 half of that is the receptors for the product. And I
21 wondered if you had looked therefore at protective or
22 influences that would tend to indicate exacerbation

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1 if you combined your analysis of variants of the beta
2 receptor genes with those of the COMT to see if there
3 are instances where there are particularly bad or
4 particularly favorable outcomes.
5 DR. MAIXNER: Right.
6 DR. CLARK: And my second question is
7 actually did you see any -- are you anticipating any
8 problems with bronchospasm and pulmonary problems if
9 you go too far in antagonizing beta 2?
10 DR. MAIXNER: The latter question first, it
11 could very well be that side effects would be the
12 same as propranolol with respiratory capacity given
13 the cellular data that beta 2 antagonistic properties
14 are about equally potent between propranolol and both
15 the r- and s-enantiomer of bupranolol.
16 So this could become a factor for selection.
17 Current day thoughts on low dose beta blockers and
18 asthma are changing. Obviously, exercise-evoked
19 asthma is a problem, but the issue of non-exercise-
20 evoked asthma, it appears that beta blockers are
21 being used commonly now. And Cochrane Reviews are
22 supporting the use in asthma without exercise-induced

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

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

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1 They want to make sure that there's intellectual
2 property that can be held. The second is big PhRMA
3 likes to see something that's already been in man and
4 to progress it. I think it has a profile, and I
5 think it has potentially a market, not only as
6 monotherapy for chronic pain but potentially as an
7 opioid sparing agent in the postoperative area.
8 So I think that it's unknown. I think there
9 are a lot of attractive features, and there are some
10 detractors from a big PhRMA perspective. Smaller
11 PhRMA may have interest in this and be willing to go
12 after a 5, 10 or something like that.
13 DR. BASBAUM: [Inaudible – off microphone.]
14 (Whereupon, at 12:57 p.m., a luncheon recess
15 was taken.)
16
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18
19
20
21
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1 AFTERNOON SESSION
2 (2:02 p.m.)
3 Q&A and Panel Discussion
4 DR. BASBAUM: So in addition to the speakers,
5 we have Sharon Hertz, and maybe we should begin by
6 allowing Sharon to introduce herself and tell us.
7 I'm Sharon Hertz. I'm deputy director for
8 the division of anesthesia, analgesia and addiction
9 products here at FDA.
10 Do you guys need anything more than that?
11 No? Okay.
12 DR. BASBAUM: You need to know that
13 everything you're going to say is going to be on
14 tape.
15 (Laughter.)
16 DR. BASBAUM: So thanks, everyone, for coming
17 back.
18 In order to get the ball rolling, I asked
19 everyone on the panel to prepare a question or think
20 of a question for someone else on the panel, and then
21 hopefully, we can evolve from there. So it should be
22 freewheeling. Don't be shy, and let's see where this

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

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

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

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

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1 yourself on the beta 1, beta 2, beta 3, I thought
2 that was superb. But I worry there that I don't
3 think PhRMA will take that forward because they will
4 be worried about the effects in asthmatics or COPD
5 patients, and the industry has become so risk averse.
6 So my question, Allan, is how do we pick that
7 key target? And I don't know.
8 DR. MCMAHON: I'll just come back on that a
9 second. I mean, one of the points of doing an
10 unbiased screen is that you're unbiased about what
11 you want to see. But nonetheless, when you see a
12 whole lot of hits that don't mean anything to you,
13 you think, well, that's a bit disappointing. Where
14 do I go from here?
15 So seeing some old friends in that list is,
16 of course, reassuring. It makes you feel that the
17 process is at least appropriate, can pick out things
18 that we know are involved at some level in some of
19 the processes we're interested in.
20 But I agree with you, that the tough bit is
21 all of these targets that don't mean anything to you,
22 how much time and energy are you going to invest in

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1 those because it's almost inevitably going to be for
2 an academic at least several years' work to really
3 make an progress against each of them.
4 DR. PORRECA: Well, just to speak to your
5 point, Steve, so isn't this -- once you see these
6 potential targets that you don't recognize and you
7 don't know what they mean, isn't this where the
8 crowdsourcing concept really would have its impact,
9 where people may be looking at this list with
10 completely divergent concepts of how and why they may
11 be interested in that particular target?
12 I would think that it can't be an individual
13 effort. It really has to be something that's broad,
14 that it calls upon the creativity of the whole
15 community.
16 DR. MCMAHON: But it's quite difficult to
17 publish the list as a fishing list, actually. There
18 should be better opportunities to do that, but it's
19 difficult to just say, here's a list of the data, let
20 other people go and mine it. You nearly always have
21 to make some kind of story up around the list.
22 DR. BASBAUM: Bill?

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1 DR. MAIXNER: I think one of the actually
2 most difficult aspect of this from my perspective is
3 trying to find the signal, the signal amongst the
4 noise where a given variant or a given pathway may
5 only contribute a small effect to the overall
6 phenotype. And I think there are two areas that need
7 to be developed that will help identify signals.
8 One is more detailed and substantial
9 phenotyping of populations, populations of interest
10 where the phenotypes are tapping into the
11 pathophysiological processes that contribute to the
12 condition of interest.
13 So I think the ability to detail in a
14 quantitative manner in several of the domains that
15 I've shown and then taking those data and developing
16 the strata, the subpopulations where you take a very
17 heterogeneous population of, say, fibromyalgia
18 patients, and then through various procedures like
19 cluster analysis, many others, you develop
20 subpopulations, which are more homogenous with
21 respect to their underlying pathophysiology. They're
22 giving these complex presentations.

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1 So I think one of the first things that needs
2 to be done is population-based studies where the
3 population is well phenotyped and then they are
4 stratified based on those phenotypic portraits and
5 then contrasting those different subpopulations for
6 molecular profiles.
7 I think that when we start to reduce the
8 heterogeneity, we'll find that a given variant has a
9 much stronger effect size and that we're now finding
10 pathways, not single genes but multiple genes that
11 are contributing to fundamental pathways that are
12 controlling the expressions of the phenotypes in
13 these subpopulations.
14 So I think one way to increase signal
15 production is to decrease the noise, and they have
16 several populations where that's decreased.
17 Then lastly, I think comment to this would be
18 the need for the development of bioinformatics tools.
19 Our pathway analyses are fraught with problems,
20 statistical problems, conceptual problems. They're
21 gleaning some information for us. But being able to
22 take large arrays of data, big data, and reduce it

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1 into pathways using bioinformatics tools is sorely
2 needed and I think further increases the signal that
3 you can see in these strata that are developed.
4 DR. BASBAUM: Steve, I do want the audience
5 to ask, and they always have priority.
6 DR. EDVINSSON: Lars Edvinsson from Sweden.
7 Concerning the TRPV aspect, I wonder if it's not a
8 little bit overstated. You need to go to -- have
9 some kind of a migrant model to look on it. And when
10 this was done, it turned out that the amount of CGRP
11 that was released from the trigeminal system was
12 rather small. So its inhibition was not like during
13 a real migrant attack, and that could be a reason why
14 when they tried blockers against that mechanism, it
15 had not a significant effect.
16 So it's important to have a good model that
17 represents the disease. So that's very important, I
18 am sure.
19 The other thing we learned from the CGRP
20 story is primates have a different kind of CGRP
21 receptor. So with the CGRP blockers, the initial
22 ones, they were equal sensitive like CGRP A237, but

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1 the new ones, they were much more potent on primates
2 compared to rodents, for example. So that's another
3 kind of interesting aspect.
4 That was my small comments here. So I agree
5 with you that it's important to look on patients or
6 primates at least.
7 Your nice data, Laura, on the disks and the
8 ingrowth of the PGP nerves, very nice. Do you know
9 what kind of fibers they are?
10 DR. STONE: That's a great question. We
11 don't know. We know that many of them are CGRP
12 positive, but we're right now doing the studies to
13 find out also if there's sympathetic fibers in there
14 or if there's IB4 positive or P2X3 expressing and so
15 forth. But it's a great question.
16 DR. BASBAUM: I think there was a question,
17 it was kind of directed at Jordana, and it got
18 shifted somewhat sideways when we were talking about
19 TRPA1. And I wonder if I could elaborate perhaps
20 because you were asking -- you said is it a plausible
21 pain therapeutic target.
22 The question I'm curious about, and I don't

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1 know. It's not a rhetorical question. I did ask
2 about the asthma. The approach that you took was to
3 do the epigenetic-wide analysis, and then came up
4 with A1. If you go the other route and say in twin
5 studies, are there differences in the incidence of
6 pain associated with particular
7 diseases -- arthritis, headache, migraine, et cetera,
8 et cetera -- and then look for differences in the
9 cohort that is already distinguished by a clinical
10 entity -- is that being done in the pain domain?
11 The question is, of course, would you come up
12 with A1 under those circumstances as well?
13 DR. BELL: So I think in the twin sample that
14 I presented, the Discovery sample, we were very
15 careful to document what other traits and whether
16 they were not on pain medication, for example.
17 So going back to asthma and other related
18 traits, there would be some cases, but those would be
19 representative of the levels that you see in the
20 population.
21 I mean, related to the comment that is it a
22 target, I can say it's a marker from our results.

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1 How you go forward, then I think you need to address
2 it with experimental --
3 DR. BASBAUM: I'll probe a little more
4 deeply, and maybe it's unfair because I'm coming from
5 a pain world. A1 is in the animal literature
6 implicated, as you mentioned, and some people think
7 it's involved in cold pain sensitivity and maybe in
8 injury associated, and certainly in irritant
9 receptor.
10 The one thing I don't think there's any
11 evidence for is a heat sensor. And I was curious why
12 you decided to use heat as your QTL or your
13 measure -- oh, this is going to blame --
14 DR. MCMAHON: No, actually, we did have a bit
15 of a disagreement about phenotyping the cohort. I
16 think as Jordana said, this was part of a bigger
17 effort to phenotype 2 and a half, 3,000 individuals.
18 And I was very keen. We did a more exhaustive
19 phenotyping, which would have allowed us to look at
20 secondary hyperalgesia, sensitization.
21 But actually, we just didn't have the
22 resources to spend that long phenotyping them. So

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1 the choice of heat pain was entirely pragmatic, and I
2 agree, arguably, not necessarily the best for a quick
3 test, either. You can argue about that.
4 Once you've got that phenotype, though, are
5 you surprised with TRPA1? Well, as it happens, I
6 would have thought in other contexts, TRPA1 is an
7 excellent target, actually. And I'm sure many drug
8 companies are pursuing it.
9 In this one to say, well, does it make sense
10 for heat pain? It doesn't superficially, but I
11 notice again in the latest edition of Nature
12 Neuroscience, there's an article that talks about
13 small variants. So many non-mammalian species,
14 TRPA1, is a heat sensor, actually, snakes. Small
15 variants in TRPA1 confer heat sensitivity on TRPA1.
16 So you're not a million miles away.
17 Then secondly, there's, I think, quite an
18 interesting story now that TRPA1 and TRPV1 form
19 heterodimers, quadromers. And so if that's true, the
20 properties of those mixed receptors are really
21 unknown. I mean, they're likely to be a hybrid, and
22 of course, TRPV1, everyone agrees, is a good heat

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1 sensor.
2 So I think asking what TRPA1 might do in the
3 context of I'm assuming sensory neurons -- it doesn't
4 have to be there. It could be in the keratinocytes,
5 which have low levels as well. Asking whether or not
6 it might be playing a role as a heat sensor in a real
7 cell in situ rather than in some hydraulic system I
8 think is a different question.
9 So I know that the knockout data doesn't
10 support a role for heat pain sensitivity, but I think
11 there's a bit of wiggle room about it where you might
12 say it's associated with, or a modified form of TRPA1
13 may actually form a different kind of interaction
14 with TRPV1.
15 So there's lots -- I agree. It's this issue
16 of cause and effect. We don't have -- you can't tape
17 that in these populations, but it's not inconceivable
18 that it's true.
19 DR. BASBAUM: Sharon?
20 DR. HERTZ: Can we get a follow-up on that
21 or -- because I have a different --
22 DR. BASBAUM: New questions are good, too.

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1 DR. HERTZ: So I think that this morning has
2 been very exciting for me. I'm not in your caliber
3 of sophistication when it comes to this area, and it
4 was really informative.
5 Some of the questions that occurred to me as
6 I was listening to the presentations regarding the
7 future and pain therapeutics was a lot of the
8 mechanisms that seem to be epigenetic that are
9 influencing the phenotypes are nonspecific to this
10 condition and, in fact, may be very adaptive to other
11 epigenetic phenomena that are occurring.
12 So what promise or what opportunity do you
13 think there may be ultimately for identification of
14 ways of intervening or influencing the system that
15 can limit some of the effects to where we're
16 interested? It's a question that is in every -- it
17 spans well beyond this particular approach to
18 therapeutics, but I think in this one, it could be
19 perhaps even more challenging.
20 Has any of the work done so far suggested
21 some possible ways of targeting this system of
22 mechanisms that are responsible for the development

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1 of chronic pain states, the hyperalgesias, all the
2 problems that we encounter?
3 DR. MCMAHON: I'll just make a comment. I'm
4 not sure in the pain field whether -- I mean, there
5 are kind of several things going on. Some people are
6 looking for processes, which are pretty much
7 exclusively and highly selectively in operation in
8 nociceptors or in pain signaling part of the nervous
9 system, and they're trying to target those. And I
10 think you could argue that some of the sodium
11 channels are expressed peripherally in which
12 mutations leading to a loss of function do result in
13 analgesia. They are great examples of a very
14 selective target that's unlikely to have side
15 effects.
16 But I would point out that none of those so
17 far have been successful in the clinic, whereas I
18 think there is a surprising amount of success, or
19 claimed success, around very nonspecific things,
20 kinase inhibitors. There are some classes of
21 compounds you'd think would be hopeless to treat
22 anything, and yet that's not my understanding.

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1 Maybe you can comment, Chas, as to the
2 successes that industry has seen with those
3 nonspecific targets.
4 DR. BOUNTRA: I agree. I mean, I think,
5 well, first of all, we can get hopefully better
6 therapeutics by specifically targeting specific
7 targets. That makes sense. Instead of having
8 nonselective molecules like the HDACs, if you can get
9 real potent selective ligands, that could be one way
10 forward.
11 I think the second thing is -- and touching
12 on Steve's comment -- if we think of things that do
13 work in pain, opioids, NSAIDs, et cetera, the
14 mu opioid receptor is everywhere. It's in the GI
15 tract. COX-2 is up regulated in many systems if
16 they're stimulated, et cetera.
17 So I know we've again been seduced by things
18 like SNSs and the AV1.879, et cetera, very target
19 specific proteins, but I don't think from a drug
20 discovery perspective we always need that. And I
21 know it's about risk/benefit.
22 I also think that sort of -- I often think in

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1 terms of neurobiology. If you've got neurons and you
2 expose them to some sort of nerve injury or tissue
3 damage or ischemia or whatever, the response of the
4 neurons is very similar. They'll sort of start
5 firing crazily. Sodium channels will go up,
6 et cetera, et cetera.
7 That's probably why things that, say, for
8 example, dampen neuronal hyper-reactivity, they may
9 work in certain pain states. They may work in
10 epilepsy. They may be neuro protective in sort of
11 stroke conditions or whatever.
12 So I think there are mechanisms that
13 translate across disease areas, if you like,
14 especially in neuroscience.
15 DR. BASBAUM: Dave, I think you might have a
16 comment on this.
17 DR. CLARK: So I might. So my comments are
18 taking this from a slightly different angle. I think
19 there are two opportunities. One is when you give
20 the drug. So not all pain is acute. Most of these
21 problems we're talking about are rather chronic.
22 But what I spoke about this morning was pain

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1 that begins at the time of surgery, which is a very
2 predictable thing. There if what we're trying to do
3 is to use the epigenetic agents to prevent
4 plasticity, which is ultimately supporting these
5 longer-term or relatively persistent states, we might
6 have an opportunity to use something that would be
7 suboptimal for a huge population for a long term in
8 the acute setting for several days, as we tend do in
9 hospitals and around the time of surgery. So there
10 we might profitably pursue something not suitable for
11 the longer term.
12 The second is I think targeting is a very
13 important part of things, and I know we're going to
14 have some talks about that later. So the other thing
15 we can do is use some of these agents that might not
16 be welcome to do their thing in every cell in your
17 body. But if delivery can be to those cells, which
18 are more involved through a virus, through any kind
19 of targeting strategy, that might help us use the
20 overall strategy more effectively.
21 DR. BASBAUM: I wonder if I could follow up
22 with a bit of a corollary on that comment, and it's

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1 just something that has -- and it bears a little bit
2 on Chas' perspective, the whole point of targeting.
3 I happen to personally believe that there's
4 an enormous opportunity for spinal delivery of drugs
5 that already exist, and one of the best examples are
6 obviously opioids. But we could go through a whole
7 list. If Tony Yaksh were here, he could probably
8 give us a list of 20 different compounds that when
9 administered to the spinal cord have a much better
10 therapeutic window than when given systemically. And
11 a lot of the failure is due to systemic adverse side
12 effects, not because the drug doesn't work.
13 Yet now, we're faced with the problem of
14 money. Why is big PhRMA not interested in a
15 spinally-delivered, good molecule? Well, because the
16 market's not big enough. And I'm curious how that
17 would affect your philosophy when I still believe
18 that the rainbow, the pot of gold at the end of the
19 rainbow, is a big driver of whether or not PhRMA is
20 going to get interested in a compound, because I
21 personally believe that the spinal cord is a terrific
22 target, and you can't get people interested in it.

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1 DR. BOUNTRA: I suppose, Allan -- I mean, I
2 honestly feel that the current PhRMA model is broken.
3 I think many people accept it. It's going to be hard
4 to change, and we can have long discussions about all
5 the things that are wrong with it.
6 I think with the model that we were proposing
7 this morning -- if, say, for example, we generated a
8 molecule and you went off and showed that it worked
9 in a very small subset of patients, but it worked
10 beautifully, and big PhRMA was not interested in
11 taking it all the way to the market, I guarantee that
12 we can get money from either the public funders or
13 the charities or philanthropy to make that happen.
14 If that's not the case, then I'll give up
15 today. But I honestly believe that that's a
16 possibility.
17 DR. BASBAUM: I'll remember the promise. I
18 think that's great.
19 (Laughter.)
20 DR. BASBAUM: Anybody else want to comment on
21 that?
22 DR. MCMAHON: Just on the issue of whether

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1 epidural or spinal deliveries are feasible. I mean,
2 it is striking that the devices companies like
3 Medtronic, they now are selling \$2 billion a year of
4 implantable devices. So I think a lot of the
5 prejudice against putting anything in these dangerous
6 spaces is just that. It's prejudice.
7 So I can imagine a world where people would
8 be more open minded to these unusual routes of
9 delivery. Don't know what the regulators would feel,
10 presumably want to be reassured. But as I say, at
11 least we have quite a bit data set now coming from
12 the devices people about the incidence of adverse
13 effects.
14 DR. BASBAUM: Somebody, unless somebody in
15 the audience would like to ask a question? Laura,
16 it's your turn.
17 DR. STONE: First, I just want to comment on
18 that because with spinal delivery, it's not just the
19 drugs that are already in clinical use that you can
20 get a better therapeutic window. We know if you give
21 them spinally, that a lot of very effective targets
22 are not being developed because we can't get drugs

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1 into the CNS. So you get around that problem, and
2 the world just opens up to all kinds of different
3 targets and types of molecules that we couldn't use
4 otherwise we have to give them systemically.
5 So my question is for Bill. And that is, so
6 you had a lot of really strong evidence that beta
7 blockers are going to be useful analgesics. And you
8 also have massive data sets from your cohorts, where
9 I'm assuming that you've asked them what drugs
10 they're on, right? And a lot of people are on beta
11 blockers.
12 So I'm curious if you've gone back to the
13 data and if your data would have predicted, just by
14 looking at who's on what drugs, this analgesic
15 effect.
16 DR. MAIXNER: Yes, thank you, Laura. We have
17 access to a few cohorts, some that we've collected,
18 so the OPPERA cohort, which now is about 4,000
19 individuals, aged 18 to 34. And now we have
20 OPPERA II, which is going to go up to 60. And we
21 have followed OPPERA I longitudinally for about six
22 years being very deeply phenotyped in the domains I

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1 showed at baseline and then following some for
2 conversion to caseness.
3 During that process, we have collected data
4 on medication usage. In fact, at three-month
5 intervals we have on a portion of the cohort
6 information related to medications. We have baseline
7 treatments if they're a pain patient. We also at
8 these quarterly updates have information related to
9 treatments that they're involved with.
10 We have not yet looked at data of drug or
11 treatment by baseline characteristics, but we'll do
12 so in this.
13 So there are other data sets available, as
14 talking to Chas at the lunch break. The cancer
15 registries in particular have a lot of information
16 related to obviously, survival but also opioid
17 consumption, medication use. And there's evidence,
18 at least for the beta adrenergic system, that
19 individuals on beta blockers have longer survival
20 times. They have reduced opioid consumption compared
21 to those not on beta blockers.
22 So I think there's some convenience cohorts

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

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

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

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

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1 they're dirty, and Steve, you certainly highlighted
2 that fact.
3 How concerned should we be about their use in
4 preclinical studies, and what do we need to do to
5 have a little more confidence that, in fact, the
6 effects being observed are due to what we think
7 they're supposed to be doing?
8 DR. MCMAHON: Well, I mean, they're what
9 we've got so it's not a bad starting place. But I
10 think you can definitely do better. And you can
11 write to Chas and say, will you make a XYZ block.
12 As I said, we've got a methyltransferase
13 inhibitor from Chas that works in vivo. So there
14 will be better tools coming along.
15 Of course, you can always go the transgenic
16 route, and I suspect that that ultimately will be
17 cleaner. Obviously, you know the problems there.
18 It's time consuming. You're stuck with a mouse, but
19 ultimately, I think it's likely to throw more light.
20 And the power of the manipulability of targets in the
21 mouse is fantastic now, so you can turn it on and off
22 pretty much wherever you want whenever you want if

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1 you've got the money to invest in making the tool.
2 DR. BASBAUM: Chas?
3 DR. BOUNTRA: Can I put another elephant in
4 the room or whatever your phrase was, Allan?
5 I think -- and Frank and I have had this
6 conversation a few times. What do we as a community
7 need to do to persuade big PhRMA to start reinvesting
8 in the pain area? Because we know many companies
9 have literally pulled out of pain completely, and
10 they've pulled out because they've done loads of
11 clinical studies, and they've all failed and they've
12 lost a fortune, and so on and so forth.
13 Big PhRMA, at the moment, what they're trying
14 to do is reduce their risk. And here we're talking
15 about novel medicines that come from novel targets,
16 novel delivery devices, novel biomarkers, novel
17 stratification methodologies. Novel, novel, novel,
18 novel means more risk, more risk, more risk, more
19 risk, et cetera.
20 I'm just thinking, say, if Andrew Witty,
21 who's the CEO of GSK was sitting here, would he start
22 reinvesting in the pain area? And I think until we

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1 provide a clear path of this is how to develop an
2 analgesic, these are the assays, this is how we're
3 going to minimize risk, they're not going to
4 reinvest. But as a society, we desperately need new
5 medicines, so somehow I think we need to start
6 helping PhRMA to do this.
7 My concern is if I think from a drug
8 discovery perspective, we have this global pot of
9 money for medical research. And that pot of money is
10 not getting any bigger. Governments can't afford it.
11 Charities can't afford it. PhRMA can't afford it.
12 So yet we want more medicines out with that
13 pot, and the way to get more medicines out is to make
14 that pot more efficient. And so what I was
15 advocating this morning was reducing all this
16 duplication because there is massive duplication.
17 And I think we have to start somehow, we have to stop
18 competing with each other and working together to try
19 and help industry deliver new medicines for patients.
20 DR. BASBAUM: Please?
21 DR. KHALSA: Partap Khalsa from NIH. I
22 wanted to see if I could add another elephant to this

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1 growing herd that we're building. And I was
2 particularly struck by some of Laura's data on
3 exercise and how consistent it is when you look at a
4 whole series of physical activity, movement-based
5 interventions, that particularly for most chronic
6 conditions, chronic clinical conditions, they tend to
7 work close to, if not equal to, if not better than
8 most of the pharmaceutical medicines that we have.
9 And in some cases, opioid use, I think there's a
10 growing bed of evidence that suggests long-term
11 opioid use is actually detrimental to these
12 conditions.
13 So the elephant in this case is, given that
14 you've got these wide range of physical
15 activity-based interventions that seem to be at least
16 modestly effective if not moderately effective
17 overall, isn't that a source of sort of looking
18 particularly from an epigenetics approach to what's
19 common amongst all these different interventions?
20 And can't that then somehow -- sort of the inverse
21 approach here of sort of looking at a specific
22 protein and trying to figure out then what you can

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1 use it for and what target -- but sort of saying,
2 well, what seems to work and can we than somehow look
3 at the epigenetic pathways to help then identify,
4 well, what could we make that would be druggable that
5 might work in these cases?
6 It may not be receptor specific, but it might
7 have this sort of dirty effect that seems to be
8 nonetheless beneficial.
9 DR. BASBAUM: Laura?
10 DR. STONE: So one of the things, if we think
11 about epigenetics and target identification as not
12 looking for a way to target the epigenetic mechanisms
13 but using these kinds of screens that we've talked
14 about this morning to identify pathways that are
15 involved in pain -- so we might get 4,000 genes that
16 are dysregulated in the dorsal root ganglion, but
17 they might only belong to five or six pathways that
18 have to do with glutamate-related plasticity or nerve
19 inflammation or something like that.
20 So the first point that I want to make is
21 that we should be using these big data sets to do
22 this sort of pathway analysis and then go after

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1 things that are within those pathways where there's a
2 lot of convergence.
3 The second thing is if you think of the
4 global landscape, something like exercise has shifted
5 that global landscape. So if we could figure out
6 what's different in the disease state versus somebody
7 who's having therapeutic benefit from exercise, do
8 pathway analysis there, we're going to find out what
9 pathways are the positive therapeutic ones. Maybe we
10 go after them pharmacologically as well.
11 DR. CLARK: So I'll add that it's not an
12 epigenetic project, but we are taking a similar
13 approach in a model of complex regional pain
14 syndrome, which for years was studied using small
15 molecules and antibodies, and did experiments very
16 similar actually to the ones that were described
17 here.
18 Exercise is a brilliant treatment for these
19 animals. I think I can cure any rat of anything. I
20 don't know about any human of anything, but the rats
21 and mice do seem to get much better with exercise.
22 And we're undertaking the effort of looking for

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1 overlapping pathways but on a much more focused group
2 of molecules which will be easier for us to approach.
3 I think we're having some success in looking
4 at things like inflammatory mediators generated by
5 dorsal horn glial cells as one of those pathways,
6 which might not be a surprise, I suppose. But I
7 think that it will be a good idea to incorporate
8 these many different modalities to get at that core
9 group of targets that we'd hoped to find.
10 DR. MCMAHON: So as a neuroscientist, I don't
11 feel I have to kind of apologize for being interested
12 in the basic biology, and I'm happy to do that. But
13 I'm 100 percent with you that to not utilize easy
14 things that could offer a fair degree, maybe a lot of
15 protection or improvement, is crazy. But that really
16 is a political and a regulatory issue in many ways.
17 So here, if I understand it, there's the
18 attempt by New York to regulate the size of soda that
19 could be sold was overturned. It's unconstitutional
20 in some way. So I think if we don't take advantage
21 of the ordinary interventions, if we don't educate
22 people, if we don't regulate, we've only got

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1 ourselves to blame. The solution is not going to be
2 just to take a pill, to develop a pill for
3 everything. But the pills will help, can help, are
4 life changing in some circumstances, so I would agree
5 that some holistic approach is necessary.
6 I mean, but definitely, I think we could do a
7 lot by lobbying to get better regulation, better
8 enforcement of the opportunities we already have.
9 DR. MAIXNER: Just one comment on the
10 question about non-pharmacological agents. I think
11 it's really interesting that pharmacological agents
12 that we use today, the majority, if not all, don't
13 create new biological events, new processes. They
14 merely work with endogenous processes and have many
15 off target effects in general.
16 In contrast, some of the behavioral
17 interventions tap into those very same endogenous
18 processes and are largely free of side effect. So I
19 think it would be extremely interesting to really
20 dissect out in substantial detail those behavioral
21 therapies and interventions that we know to work and
22 to understand them well because I think that will

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1 help reveal therapeutic targets that could be
2 combined with some of the behavioral interventions.
3 So I think there's a real window there that's
4 not being captured that could inform us for better on
5 target effects and better targets for development.
6 DR. BOUNTRA: Could I just respond to that?
7 So maybe this comment's better saved for tomorrow
8 afternoon, but I sometimes think as a community we
9 need to be a lot slicker and faster. So I think of
10 the anti-CGRP approach for migraine. I know
11 Boehringer Ingelheim had some early clinical efficacy
12 data at the start of this century, so now 14 years
13 later, we still actually don't have a molecule on the
14 market.
15 If I think of the NGF approach, I think some
16 of the efficacy we've seen in patients -- and David's
17 here. He can tell us what we're saying wrong. But
18 it's absolutely remarkable. And from an efficacy
19 perspective, that's one of the biggest successes.
20 Now, I know the preclinical literature on NGF
21 blockade or TrkA antagonist was -- Frank, I'm looking
22 at you because you know it all -- 15, 20 years ago at

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1 least? And we still haven't translated that into
2 patient benefit, et cetera.
3 The sodium channel blockers, I was working
4 with John Wood in 1990 on a DRG-specific sodium
5 channel, and 24 years later, we still don't have a
6 drug on the market.
7 So somehow even with these really high
8 priority targets, I don't know how we can rally round
9 and make it happen.
10 DR. BASBAUM: I don't even know what to say.
11 I really think that's two ships passing in the night,
12 the fact that there isn't a good NEV1.8 blocker
13 because some of the animal data predicted is not
14 because -- and everyone went after it. I'm not quite
15 sure what you're saying that they went about it the
16 wrong way? It's a very difficult drug to target
17 selectively. That's one of the first problems. We
18 know that.
19 The anti-CGRP story is a complex one. That
20 was small molecule when it started, and now the -- as
21 you know, Merck had a small molecule that
22 unfortunately had an off target failure in liver, and

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1 now its antibodies to CGRP.
2 These things take time. It's very different
3 from the cancer world where tox is less of a concern,
4 unfortunately, but it is when you're trying to kill a
5 cell. If you have some normal cells get killed, you
6 might tolerate it a little bit more.
7 So I think there's a little bit of throwing
8 babies out with bathwater here to dump on the way
9 that the science and the development has occurred. I
10 think you're sort of looking at the cup really half
11 full.
12 DR. BOUNTRA: No, no, no, not at all, Allan.
13 All I'm trying to say is we have these clinical
14 hints, and we're desperate for new analgesics. So
15 how do we make it go faster? And I'm just trying to
16 think, are there lessons we can learn?
17 So that was it. I mean, so with CGRP we knew
18 at the start of this century, albeit not with an
19 optimal molecule, that there was potential for
20 clinical efficacy. All I'm saying is 14 years later,
21 that's not translated into patient benefit.
22 The sodium channel blocker, I know absolutely

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1 all the issues around tractability and selectivity
2 and so on and so forth. And maybe we don't even need
3 a selective molecule, but we've been working on it
4 for 24 years. That's a long time.
5 The NGF, Dave Shelton's doing a brilliant
6 job, I'm sure, but I worry about will PhRMA take it
7 all the way because they might be afraid of being
8 sued for osteonecrosis.
9 DR. BASBAUM: That's a good place to end.
10 (Laughter.)
11 DR. BASBAUM: Misha, I think you were going
12 to give us a little more lighthearted approach,
13 please.
14 DR. BACKONJA: I don't know how lighthearted
15 it's going to be, but at least put some light on the
16 perspective. As a clinician scientist going to
17 meetings and seeing so many targets and mechanisms,
18 one of the things that was always leading me with
19 like which one matters -- and what really was primary
20 impression of these series of presentations -- is
21 that finally, we're talking about really what's
22 relevant in the clinical world is the fact that

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1 there's a time course to everything that's happening,
2 and that now there are possibly or potentially ways
3 to start prioritizing these mechanisms.
4 So that was a probably high point of my
5 realizing what's going on here, but I'm still not
6 convinced which of these approaches is the way to go.
7 And I don't know if anybody dares to step out and
8 tell us that.
9 DR. MCMAHON: We've obviously got no idea,
10 either. That's why we're doing these.
11 (Laughter.)
12 DR. BASBAUM: I was going to turn it to the
13 audience, and Roy, let me ask you a question, if I
14 may.
15 So Roy Freeman is a neurologist, who you have
16 done -- and others in the audience who have done
17 comparable studies, Misha included, clinical trials.
18 So now we've gone through the preclinical phase.
19 It's gone through phase 1. And now someone
20 approaches you and say, we want to take this to the
21 patient, PHN trial.
22 To what extent do you sort of look at all the

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1 previous work to decide whether do I really want to
2 take the time to do this work? You must have a lot
3 more confidence in the compound than Chas has that
4 you're taking it in and it may fail.
5 I'm using -- I'm sorry, Chas, but it's easy.
6 Roy, any thoughts?
7 DR. FREEMAN: I lost you with the question.
8 As soon as you brought Chas in, I --
9 (Laughter.)
10 DR. BASBAUM: No, the question is somebody
11 says, Roy, we'd like you to take this compound into
12 the clinic and test it in X. And you are probably
13 making some decision as to what the target should be.
14 I don't mean the site, but the patient population.
15 And you're building that decision on the preclinical,
16 what they've told you, the phase 1 data
17 Do you ever sort of take the perspective that
18 I'm probably wasting my time because it's probably
19 going to fail?
20 DR. FREEMAN: Well, I go along with the
21 series of points -- the continuum of points that Chas
22 showed on his slide of which things fail. You've got

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1 to be a realist over here and say that things are
2 going to fail at every point along the way and then
3 just -- even if we get to phase 3 and they work,
4 there are probably going to be toxic side effects. I
5 think that's the nature of the business.
6 If the question was -- which I thought it was
7 going to be -- what do we want preclinically, what
8 kind of preclinical package will give us some clue, I
9 have the same nihilistic attitude there as well. I
10 think we want some clue that it is going to work
11 preclinically, that there is some preclinical
12 evidence. We want some clue from the models.
13 But I think we can't put too much weight on
14 it, and I think tomorrow afternoon's session gives us
15 some sense as to how many molecules have actually
16 moved from the what we think of bench to bedside
17 route. And you can count them on much less than one
18 hand, unfortunately.
19 DR. STONE: If I could comment on that?
20 There's a couple of things that we can do in the
21 preclinical community, and one of them is if we're
22 going to model chronic pain, then we should model

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1 chronic pain and not inject CFA and test the animal
2 24 hours later and call it arthritis. That's not
3 chronic pain. You have to wait for a long time.
4 Just like in people, chronic pain takes a long time
5 to develop and is very intractable once it develops.
6 So why are we not waiting three months after
7 nerve injury or joint inflammation to test our drugs?
8 Because the system is going to be completely
9 different three months later than it is a week later.
10 The second comment is that in the preclinical
11 world -- and I think we're all guilty of
12 this -- we'll take some animals, and we'll inject a
13 drug once. We'll test the animal an hour later, and
14 then we'll make conclusions about its efficacy. You
15 would never do a clinical trial like that, right?
16 Why are we not giving the drug for a month in the
17 animals?
18 In the exercise study that I showed, if we
19 had stopped looking after a week, we never would have
20 seen what I believe are disease modifying effects of
21 running exercise because they weren't there after a
22 week. It took a couple months. So that's one

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1 suggestion.

2 DR. BASBAUM: Frank?

3 DR. PORRECA: Well, I just had to comment a

4 little bit on Roy's point about the preclinical

5 studies and just remind everybody that much of what

6 we know about nociceptive pathways and modulatory

7 pathways have come from the preclinical science. So

8 saying we shouldn't put too much weight on this, I

9 think is not right. That's the first thing.

10 The second thing, the point was made about

11 spinal administration. And I just want to circle

12 back to that for a moment and just remind everybody

13 that the correspondence between preclinical

14 evaluation of reflex responses with spinal

15 administration and efficacy in humans with spinal

16 administration is essentially 100 percent.

17 So the point is that the biology

18 translates -- and I think we've got a talk that's

19 coming tomorrow by Toto Olivera that I think is going

20 to remind us about this, translation of biology

21 across species, the mechanisms translate.

22 Now, making a drug is a different question,

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1 it's a hard question, for systemic administration.

2 But I don't think you can question the fundamental

3 relevance of biology, and that's something that we

4 shouldn't forget.

5 DR. FARRAR: Yes, John Farrar, University of

6 Pennsylvania again. Frank, I agree with you

7 100 percent, that the biology has been spectacular in

8 terms of dealing with earlier models.

9 I do have to say that I also couldn't agree

10 more that when we get to thinking about clinical

11 effectiveness, whether we think we're about clinical

12 efficacy, we do need to study chronic pain models.

13 And there are some chronic pain models in animals

14 that certainly could serve as potential useful

15 treatments. Yes, we probably need to study them

16 longer.

17 But I wanted to actually get back to the

18 point that you raised with Roy, which is what about

19 moving this forward into people. And one of the

20 things that I think that has come out of the IMPACT

21 ACTION process has been an understanding of the way

22 in which we should move forward with proof of concept

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1 trials.

2 That's obviously affected by a push from

3 industry to get done faster and to try and combine

4 the dose finding and proof of concept in the same.

5 But if you take a step back, one of the things that's

6 true from everything I've heard today is that the

7 population that's likely to be benefitted by any one

8 particular entity that we've looked at today is going

9 to be relatively small.

10 Identifying that group a priori in a large

11 population of patients with back pain is nigh short

12 of impossible at least currently. One way around

13 that is to do and to start out with proof of concept

14 trials that are either randomized withdrawal or

15 enhanced in some other way.

16 Randomized withdrawal trials, there's a long

17 history of using them. They're not perfect. They

18 certainly don't serve as the only trials that you can

19 do to get approval. But it gives you the opportunity

20 to use your drug, to identify the patients that are

21 likely to benefit from that drug, and then to see

22 whether if you take it away and give it back to them

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1 or just take it away in a randomized way, whether you

2 can demonstrate that they actually saw a difference.

3 It gives us the ability within a large

4 population to identify at least one subpopulation

5 using the drug to identify one that benefits. If you

6 can't prove it there, then it's time to give up.

7 But it seems to me that we waste -- in the

8 past, we certainly have wasted a fair amount of time

9 going from sort of small randomized trials parallel

10 groups to large randomized trials to show that it

11 doesn't show anything. And so I wondered what the

12 panel might think about that and what others in the

13 audience might think.

14 DR. BASBAUM: If the panels wants to comment,

15 and then I think we will take a break because we're

16 going to continue after. We have a whole day

17 tomorrow.

18 DR. MAIXNER: I'd like to comment. And if

19 there is any breathing room for one last question,

20 I'd like to pose a question to the audience, too. We

21 have a few minutes.

22 DR. BASBAUM: Minute and then the question.

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1 DR. MAIXNER: The comment to John's thought
2 is I think that this is maybe one of the main reasons
3 why there's failure from phase 2 to phase 3, that if
4 you look at the way that most clinical trials are
5 designed in phase 2, it's to select individuals who
6 have only that specific condition.
7 For example, many of the fibromyalgia trials,
8 individuals were recruited, selected, who only have
9 fibromyalgia. Well, that's a rare bird. Most
10 chronic pain conditions, 15 percent or less only have
11 that select condition. The vast majority have other
12 comorbid conditions.
13 So I think that we have a selection bias that
14 is working against us. And so I think I advocate for
15 case definitions and inclusion of individuals who are
16 more common to the clinic. And I think that that
17 will promote success, and I think we'll then entice
18 PhRMA further, not all the way, but further into the
19 process when there's success in larger portions of
20 the population rather than the 30/30/30 rule. And
21 that will continue as long as we limit the nature of
22 the population that we try a randomized control trial

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1 on.
2 The question I'd like to pose to the group
3 relates to the theme of the workshop today,
4 epigenetics. We as a group do very well at
5 characterizing pain populations, genotyping them, the
6 methodology is great. What I think we're lacking
7 here are good instruments that assess the
8 environmental exposures that individuals have.
9 So I'm curious from the audience perspective,
10 what instruments should IMPACT or ACTION be thinking
11 about that can capture the key environmental
12 exposures that will lead to stratification
13 capabilities, that may help us inform us as to why an
14 individual may or may not respond to a drug. So we
15 have good ways of phenotyping, molecular profiling,
16 but I think we really have a long way to for
17 assessing environmental exposures.
18 So what instruments should be used?
19 DR. BASBAUM: Sounds like a good topic for a
20 next meeting because no one has the answer yet, and I
21 think people are looking for a cup of coffee. So
22 we'll sit on that one.

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1 Thank you all. Thanks to the panel and
2 everyone. We'll be back in an hour.
3 (Whereupon, a recess was taken.)
4 DR. PORRECA: All right. We're going to get
5 started on the afternoon session, and I think we had
6 a very lively and interesting discussion this
7 morning. And I'm very pleased to move on to the
8 topic of imaging in translation.
9 I think that one of the things that emerged
10 from the morning discussion is the concept of
11 de-risking targets and investment and how we can
12 increase confidence. And I think that one of the
13 things that didn't come up that is a barrier is
14 exactly how we measure pain.
15 So we all know the complexity of pain and the
16 subjective characteristics of pain in individual
17 patients. And so when we think about investing in
18 development of pain therapeutics, as opposed to
19 investing in some other area of biology where there
20 is an objective outcome measure, one can make the
21 argument that it's a safer bet to go with a disease
22 that would have some sort of an objective readout.

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1 That's not the case in pain at least at the
2 moment. I think that things are moving forward into
3 gaining better insight into how we actually evaluate
4 pain, and imaging has played a tremendous part in
5 this.
6 So it's a pleasure to introduce Catherine
7 Bushnell, who's going to give the first talk entitled
8 From Mice to Men, the Role of Brain Imaging in
9 Translational Pain Research, which I think is exactly
10 the topic.
11 Catherine is the senior director of the NCCAM
12 here in Washington and was the director of the McGill
13 pain center in Montreal. Thanks, Catherine.
14 Presentation – Catherine Bushnell
15 DR. BUSHNELL: Okay. Thank you, Frank.
16 But I'm afraid if you want somebody to say
17 that imaging can tell that somebody's in pain, you
18 should have invited Irene and not me.
19 (Laughter.)
20 DR. BUSHNELL: But I will try to talk about
21 how we can use brain imaging as a tool and possible
22 translational research.

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1 So I have nothing to disclose, but I'm
2 obliged now as a government employee to tell you that
3 these are my personal views and not that of the NIH,
4 or the Public Health Service, or the Department of
5 Health and Human Services.
6 So I just want to start off with some of the
7 reasons why one might want to conduct parallel brain
8 imaging studies in animals and in rodents as
9 preclinical models. And one thing is we have these
10 clinical observations and the ability to kind of back
11 translate into our animal models and to then look at
12 possible mechanisms of this that imaging can make the
13 link between the clinical observations and being able
14 to go back to rodent models and see, using a parallel
15 technique as a whole brain imaging to see if there's
16 similarities at that level.
17 Also, as we're developing mini genetically
18 modified animals and people are looking a lot in the
19 spinal cord at what's happening in these animals to
20 be able to get an overview, a whole brain overview of
21 possible changes in the pain system or modulatory
22 systems so you know where in the brain to be possibly

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1 targeting more detailed analyses. So this gives you
2 kind of a whole brain overview that can be useful.
3 And together these things maybe can lead to better
4 translation between preclinical models.
5 So I'll go through and give examples of
6 different types of imaging that's involved in human
7 studies and how it's starting to be applied to rodent
8 studies, and then come back to whether or not we can
9 actually use these things successfully.
10 So just an overview, we all know that there
11 are many parts of the brain that are now involved in
12 pain processing in the human brain. And imaging
13 shows us that -- it's an example of just heat pain
14 standard imaging of a phasic heat pain stimulus in
15 healthy people, and seeing that in fact there are
16 multiple parts of the brain that are activated by the
17 pain stimuli. And these involve some meta-sensory
18 areas such as primary and secondary somatosensory
19 cortex and also areas in the limbic system and
20 pre-limbic system, including the anterior cingulate
21 cortex and the insular cortex.
22 So if you go into the rodents, we actually

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1 can see that both these sensory and affective areas
2 can be activated with an acute pain stimulus in
3 rodent models similar to what we see in the human
4 models.
5 I'll show you -- this is an example that
6 Scott Thompson in our lab did. Throughout this whole
7 talk, I'll be bringing up the issue, and I'll talk
8 more about it at the end, about anesthesia and stress
9 and other things that when you try to image a rat or
10 a mouse can become quite an interesting issue that
11 may not apply to the human studies.
12 So when we do human imaging studies, first of
13 all, we make sure they're not claustrophobic and that
14 they are comfortable in the scanner. At NIH, we have
15 a mock scanner that we put them in first to condition
16 them. But then you take a rat or a mouse and you
17 either anesthetize them or you tie them down. And
18 he's not going in there voluntarily and does sign a
19 consent form, and he may be having a very different
20 experience.
21 One issue when you're doing whole brain
22 imaging is you're imaging everything that that little

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1 guy is thinking and feeling, and so you're not just
2 imaging the stimulus you're putting on the skin,
3 you're imaging everything that's going on. And that
4 complicates the issue and something we have to be
5 aware of.
6 But one way to get around that is to do what
7 we've done in our lab that has pros and cons, but is
8 to do -- this is PET imaging, where we use a
9 radioactive tracer to look at metabolic activity as a
10 surrogate for neuronal activity.
11 The temporal parameters are such that the
12 uptake period is before -- you can have an uptake
13 period where the animal is just in a box, and you can
14 expose them to whatever you want to expose them to.
15 And then you can anesthetize them and put them in the
16 PET scanner, and you can see the residual from the
17 activity, the uptake that occurred during this pre-
18 scanning period. So the animal's not stressed. He's
19 just sitting in a normal environment.
20 So you can do this where -- what we did is
21 did a formalin injection into the rat's hind paw, had
22 him sit there and then during this uptake

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1 period -- of course, rats don't just sit there when
2 they get formalin. They shake their paw, and they
3 lick their paw and do various things.
4 So what you can -- and when you scan, then
5 you're going to be scanning not only the pain that
6 they may be feeling but also these behavioral things
7 that they're doing to compensate for the pain. But
8 you can measure all this. You can videotape it, and
9 you can record all of this. And then you can look at
10 what's happening in the brain during this uptake
11 period.
12 We saw found activations in the primary
13 somatosensory cortex, the hind limb area where we had
14 injected the formalin, and in the cingulate cortex,
15 CG1, but also in the face area. But then if we
16 looked at the videotapes of the animal licking and
17 shaking, we found that the licking behavior
18 correlated perfectly with the activity that we saw in
19 the face area of the cortex, suggesting that this was
20 being driven by the licking, biting behavior, whereas
21 the activity we saw in the hind limb area was
22 unrelated to the shaking -- the hind limb motor

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1 behavior, suggesting that this was not being caused
2 by the movement. And from that, we assumed it was
3 being caused by the afferent input, the, quote,
4 "pain."
5 Then the cingulate cortex that I'm showing
6 you here in the graphs also did not correlate with
7 any behavior, suggesting again that this was more
8 related to the afferent side.
9 So you're seeing these things. So this is a
10 case where the animal is in a non-stressed,
11 non-anesthetized condition, but the downside of doing
12 it this way is you do have to factor out the other
13 things that you know are going on.
14 So I'm going to go through -- there's a
15 number of imaging techniques that are used for
16 studying pain in humans. One is anatomical imaging
17 that we have the BOLD technique, the pain evoked
18 responses, the most commonly used functional brain
19 imaging technique. I'll explain all these as we're
20 going along.
21 One is resting state imaging, which is
22 something I'll explain to you and how it's going to

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1 be altered by pain. And then more recently people
2 instead of using BOLD, they're using this arterial
3 spin labeling, which gives you an absolute measure of
4 blood flow as opposed to a relative measure.
5 Then you can actually -- you can compare data
6 between scans, and you can look at a more chronic
7 condition. You don't have to have very quick on and
8 off as you have to do with using a BOLD technique
9 that requires kind of a change in signal that
10 actually has a slow drift. And so you can't look at
11 slowly occurring events. You have to look at very
12 rapidly occurring events; and then PET imaging to be
13 able to look at various radial ligands and looking at
14 changes in neurotransmitters or the chemicals.
15 Anatomical imaging. Over the last decade or
16 so, there have been many, many studies. I think it
17 was Vania Apkarian in 2004 was the first study out
18 there showing that chronic pain patients have changes
19 in their brain anatomy, and many studies find
20 small -- there's slight differences from study to
21 study. But overall, we find that people who have
22 chronic pain have decreases in the amount of gray

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1 matter, particularly in areas that are involved in
2 pain processing and pain modulation.
3 Just as a couple examples, this is from Dave
4 Seminowicz with Laura Stone, where they looked at
5 chronic pain patients and compared them with matched
6 healthy controls. And these blue areas on these
7 brains show areas where there's significantly thinner
8 cortices in the pain patients than the matched
9 control subjects. So these are age, sex, lifestyle,
10 socioeconomically matched people.
11 So you see that particularly a lot of these
12 frontal areas, dorsal, prefrontal cortex, as Laura
13 pointed out earlier, another medial -- a lot of this
14 prefrontal areas, in fact, are thinner in the pain
15 patients than in the healthy controls.
16 We've done this with fibromyalgia patients.
17 And again, you find this -- here the red shows area
18 where there's significantly less gray matter in the
19 fibromyalgia patients compared to controls. And
20 again, these prefrontal areas dominate in the areas
21 where you find thinner cortices.
22 So you can take this general finding that we

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1 have in many, many studies now in pain patients, and
2 you can back translate into your rodent model. One
3 issue that's going on with the pain patients is that
4 you don't know what's cause and what's effect. It
5 may be -- we talk about -- lots of people say with
6 back pain, lots of people hurt their backs, and most
7 people get better, and they don't go on to have
8 chronic pain, whereas other people do go on to have
9 chronic pain.

10 Well, if you go back to this, it could be
11 that after an injury, the people that just have
12 really crummy dorsal lateral prefrontal cortices are
13 more inclined to have their pain become chronic than
14 people that don't. So we don't know if these brains
15 were already different and that's a factor that makes
16 them susceptible to having chronic pain or if the
17 pain itself is leading to their having reduced gray
18 matter.

19 Now, with these cross-sectional studies, you
20 can factor in how long they've had their pain. And
21 in the studies that do that, if you use that as a
22 covariate, we generally find that the longer the

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1 people have had their pain, the less gray matter they
2 have, suggesting that, in fact, it is causative, but
3 it's not really definitive.

4 But in the animal models, you can actually
5 take genetically identical animals, and you can
6 expose them to a pain stimulus. And you can look
7 across time. And this is what we did again with Dave
8 Seminowicz in our lab, where we took rats and we did
9 an SNI model. And half of them had sham surgery, and
10 half of them had the SNI injury. And then we looked
11 over time for up to 24 weeks after the surgery. And
12 we did every week -- well, not every week, but every
13 few weeks, we put them in a little MRI scanner, and
14 we did an anatomical scan of their brain.

15 We found that, in fact, this prefrontal
16 cortex of the red glob there, which is prefrontal
17 cortex, in the animals that were injured ended up
18 ultimately being thinner than in the animals that
19 just had the sham surgery. But you see this is not
20 significantly different. These were actually young
21 animals that were growing, so their brains were
22 growing. And you see the sham animals were growing

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1 more rapidly than the SNI animals. But we didn't see
2 a significant difference until 20 weeks after
3 surgery.

4 So this goes back to this issue about doing
5 these experiments where you do an injury, and then
6 you look what's happened in the next day or week or
7 two weeks or three weeks, and it's still not going to
8 be sufficient to see these kind of changes. So you
9 get a chronic pain patient who comes in and you
10 do -- you put them in a drug trial, and their brains
11 have been changed while you do these drug trials in
12 animals where they're nice and healthy.

13 So I think this is an important thing. It
14 also allows you -- in this study -- we haven't had
15 great success with our tissue, and we're doing some
16 more studies now -- that you can then after
17 you -- people say what do these changes mean
18 physiologically in humans?

19 So you see these using anatomical MRI that
20 your cortices are less thick. And is this neuronal
21 death? Is this changes in dendritic arborization and
22 synaptic connectivity? Is this related to

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1 non-neuronal cells? Is it related to water? Who
2 knows?

3 So this has been a big issue that's not
4 really resolved, and I think this can be resolved.
5 The data are starting to suggest that this is not
6 really related to frank neuronal death but maybe more
7 related to changes in dendritic arborization and
8 synaptic connectivity. But the data, there's just
9 not much out there yet on this.

10 But this kind of model allows you then to
11 take tissue and go back and do other analyses in the
12 animals that then hopefully will inform you about the
13 human models. And again, it allows you to control
14 their environment.

15 We were talking a lot about epigenetics and
16 environmental factors. And then you can take these
17 animals, and you can make them smoke for those
18 20 weeks or you can do all sorts of things that
19 people -- make them drink Coke, liter Coke bottles
20 for 20 weeks. But you can do all the things that we
21 do to undermine our health.

22 So I think this provides a very nice model

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1 and an easy way to be able to start to understand the
2 changes you're seeing -- the anatomical brain changes
3 that you're saying with humans and human pain
4 patients. And this is easy in the sense that you
5 don't have to worry about anesthetics because you're
6 not doing functional but just anatomical.
7 Now we get into the functional brain imaging.
8 So there's this BOLD technique, which is used for a
9 lot of human studies. As I said, it requires -- it's
10 a relative signal that requires a fast on and off
11 with stimuli, so it works best with acute types of
12 stimuli.
13 There have been some studies with rats using
14 strong electrical stimuli under anesthesia, and you
15 can get something similar. But Becerra and Borsook
16 have been kind of leading the way in trying to do
17 awake imaging in rats, where they have several days
18 of conditioning, where they put them in a little
19 restraint thing, and they condition them for several
20 days, and then put them in a scanner.
21 Towards the end of the talk, I'll show you
22 what I think are some of the problems with this. But

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1 this is a study they published recently where they
2 did heat stimuli, parallel stimuli in humans and
3 rats, and they looked specifically at nucleus
4 accumbens and the anterior cingulate, and showed that
5 they got analogous responses to the pain onset and
6 pain offset in these regions in their rats and in
7 their humans. And I think using a parallel
8 technique, just showing that, in fact, you can get
9 very parallel data with using these methodologies.
10 Now, the mouse is very interesting to many,
11 many people. Now, we're getting away from rats, and
12 then if you want to start getting into genetic
13 manipulations, you want to be imaging the mouse. So
14 obviously, their brains are a lot smaller, so your
15 resolution is not as good.
16 But now the imaging techniques are getting
17 better. At NIH, we're getting a cryoprobe, which is
18 supposed to really enhance it even more. So coming
19 out of these labs now when you have 11 Tesla scanners
20 and you have cryoprobes and things, the resolution is
21 getting better.
22 This is an example of a study that just came

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1 out recently using -- it's electrical stimulation, so
2 again, it's a very strong stimulation that we know in
3 rat studies seem to survive anesthesia. And that's
4 what this is, but you can see -- but then they are
5 actually looking at the effects of a new adrenergic
6 acting anesthetic or whatever it is. I'm sure it's
7 not a classic anesthetic, but showing that, in fact,
8 that even with higher and higher -- these three
9 graphs are showing higher and higher levels of the
10 anesthetic and showing that this electrical
11 stimulation survives the anesthetic.
12 But it's saying that actually, electrical
13 stimulation can survive lots of anesthetics; but just
14 showing activation and sensory motor cortex. It's
15 showing that it follows the temporal parameters quite
16 well. So people are starting to image more and more
17 in mice.
18 So there's a whole other type of imaging
19 that's kind of come into the pain world, and it's
20 called resting state imaging. And this is something
21 that was first identified by Fox and showing that
22 when you have humans and you're doing tasks in the

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1 scanner, there's a period of time between the task
2 where you're resting. And there are brain circuitry.
3 If you look at how different parts of the
4 brain are activated in time and do temporal
5 correlations between various brain areas -- so you
6 take areas A and B and look how each of them is
7 increasing and decreasing activity across time and
8 seeing which ones in fact have a strong
9 correlation -- you find that there is a network that
10 involves primarily the posterior cingulate/parietal
11 cortex and this big frontal area, so the stuff in
12 blue and green, that are activated more when a person
13 is not doing a task than when he is. And it turns
14 off when a person is doing a task. And that's why
15 it's negatively correlated with a task, and it's
16 called the resting state network.
17 Then there are others that no matter what
18 task you're doing, other parts of the brain are
19 active whenever you are doing something, engaged in
20 something. And so people have now identified a bunch
21 of networks in the brain that are just coherently
22 across time activated or co-activate.

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1 So this default-mode network, the one that's
2 activated when you're at rest, has been studied now
3 in humans and in monkeys and in rats, and basically
4 has been identified in all three of these species.
5 And so it seems to translate across species, and it's
6 important because it can be -- chronic pain itself
7 can alter this default-mode network, And this is
8 something -- I'm giving you an example from our lab
9 from Marta Ceko's work with fibromyalgia patients.
10 What she did is she put a seed here in this
11 posterior cingulate, in this part here. And then she
12 looked at connectivity with other parts of the brain
13 and found in the healthy people and in fibromyalgia
14 patients, and found that this connectivity with this
15 anterior part is disrupted in the fibromyalgia
16 patients. They're shown in red.
17 This is actually independent of the pain that
18 they're experiencing while lying in the scanner
19 because, obviously, if a pain patient's in the
20 scanner -- like you take a back pain patient and you
21 make them lie in a scanner for an hour, they could be
22 experiencing substantial pain from their back. And

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1 so if you're looking at something that's supposed to
2 be active when a person's at rest and they're not at
3 rest because they're cursing you about the fact that
4 you're making them stay in a scanner with their back
5 hurt, then they're not resting.
6 But even if you take into account possible
7 pain that's going on by the patient while they're in
8 the scanner, you have a disruption in this default-
9 mode network. We don't know the meaning of that, but
10 we know that somehow pain is altering the way the
11 brain is functioning.
12 Again, David Borsook and Becerra have
13 identified a number of resting state networks in the
14 awake rat. And I can't exactly explain this whole
15 graph, but it's just showing -- because it's not from
16 me. But it's just showing the areas that are
17 co-activated together during a resting state with the
18 idea that awake rats can also have this resting state
19 activity.
20 But more interestingly to me is the work that
21 David Seminowicz did in Baltimore recently, where he
22 basically looked at how a nerve injury can alter some

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1 of this connectivity in the brain. And he found
2 that -- I'm just giving you one example from his
3 paper. There were a couple of other networks that
4 were disrupted. But the relationship between the
5 somatosensory thalamus and the somatosensory cortex
6 was disrupted in these animals that had a nerve
7 injury.
8 So after surgery, you see that as time goes
9 by, it becomes more disrupted. If you look at the
10 difference between the normalized to the shams, that
11 the -- this red and blue is showing connectivity
12 between the two sides of the thalamus, and the green
13 and black are showing connectivity to the
14 somatosensory cortex.
15 So you have increased -- in the nerve injured
16 animals, you have increased intrathalamic
17 connectivity and decreased thalamocortical
18 connectivity.
19 So again, I'm not sure of the meaning of
20 this, but it just shows that, in fact, in the animals
21 models, as in the human models, you can find
22 alterations. And this is not anatomical

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1 connectivity. This is functional connectivity, just
2 meaning synchrony and activation in these areas
3 that's disrupted.
4 Now, again, system analysis, it's interesting
5 this group that's in Europe that's been doing the
6 mouse is showing that functional connectivity -- and
7 again, they're using their three levels of
8 anesthetic. And interestingly here, whereas the
9 anesthetic did not alter, did not have an effect on
10 electrical stimulation, produced functional activity,
11 it does not have an effect on -- this highest dose of
12 anesthesia has an effect on these intrinsic
13 functional connectivity within the brain, as you can
14 see by this less red.
15 So it's something again to be aware of with
16 anesthetics, that this could be affecting this type
17 of activation where it survived -- the electrical
18 stimulation can survive. And this just graphically
19 shows the inter-hemispheric connectivity in the
20 resting mouse brain, and again, disrupted with this
21 highest level of anesthetic. But the lower levels,
22 it does not seem to be disrupted and looks pretty

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1 good that you can give them some anesthetics and
2 still show this inter-hemispheric connectivity.
3 So these are possible. In the mouse, they
4 haven't taken it to the level of looking at chronic
5 pain and how it's disruptive, but it looks like it's
6 a viable model.
7 So now, arterial spin labeling. If you want
8 to look at a chronic condition, you can't really use
9 the BOLD technique. So you can look at things like
10 disruption of resting state. But if you want to
11 actually look at what's activated in the brain
12 related to your pain, you either need to do something
13 like we did with deoxyglucose, or you need to do what
14 they call arterial spin labeling, which allows you to
15 be able to get a real absolute measure of blood flow.
16 Using that -- again, this is from David
17 Borsook's lab where -- and there's been several -- my
18 student Marco Loggia's done some really nice work
19 with arterial spin labeling. But people are starting
20 to use this for pain stimuli, and it seems to work
21 pretty well.
22 The pattern you're seeing here with the heat

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1 pain stimulus looks pretty much the same as you'd see
2 using the BOLD technique with activations and the
3 insula and the anterior cingulate and the thalamus
4 and hippocampus. And it looks pretty similar to what
5 you see using the BOLD technique, suggesting that, in
6 fact, this is a pretty good measure.
7 This again has been taken to the rat with
8 arterial spin labeling. We're starting to do this
9 with the mouse, so next year, hopefully, I can tell
10 you that it worked. Keep my fingers crossed. But
11 with the rat, you can see nice temporal
12 correspondence with the electrical stimulation.
13 Using arterial spin labeling looks pretty much the
14 same. The same people have done this with BOLD, and
15 it looks pretty much the same as the BOLD.
16 So the nice thing about it, with these short
17 stimuli, you don't need it. But if you want to use a
18 longer stimulus, then you -- so if you're going to
19 have a more chronic pain type of a measure, then you
20 want this is a technique that could work. So it
21 seems like it's working pretty well.
22 Then finally, imaging

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1 neurotransmitters -- and this is something that -- in
2 our lab we started doing this with -- so there have
3 been a number of studies in chronic pain
4 patients -- or with acute pain and also with chronic
5 pain patients -- that you can get alterations in the
6 availability of either opiates or dopamine in the
7 brain related to chronic pain.
8 So if you take an acute pain stimulus and you
9 give it to a healthy person, what happens is every
10 part of the -- and then you do this competitive
11 binding of -- this is diprenorphine for opiates. If
12 an area -- if the pain itself leads to the release of
13 the opiate in that area, then the receptors are
14 bound, and then you give this exogenous ligand, and
15 it can't bind. So you look at reduction in binding
16 potential.
17 So in order to -- and there's now studies
18 showing that chronic pain patients have alterations
19 in the way the brain reacts to a pain stimulus
20 itself, suggesting either that the receptors are
21 already -- they're either down regulated, or they're
22 occupied. Again, we don't know the mechanisms. So

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1 if we can recreate this type of thing in a rodent
2 model, then we can go back and we can look at
3 mechanistically what was happening.
4 But if you look, this is just showing the
5 baseline distribution of opiates in the brain as
6 identified using PET. And you see here that there is
7 high opiate receptor binding in many parts of the
8 pain system; so thalamus and anterior cingulate and
9 the amygdale. So many of the parts that -- the
10 insular cortex that we identify as being part of the
11 pain system, you have this high opiate receptor
12 binding.
13 So in our lab, we've started off -- as I just
14 said -- and this is just an example -- chronic pain
15 can alter this binding, and there's a number of
16 different studies. This is just one that I just
17 pulled out with central post-stroke pain and showing
18 that these are -- the regions in yellow are regions
19 where the binding is altered by chronic pain.
20 So in our lab with Scott, we've now started
21 doing this in rats. And so the only thing we've done
22 now is identified, taking it a step a time, as the

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1 baseline levels of opiate binding throughout the
2 brain. And now we're seeing it's a very, very nice
3 correspondence without human studies, which is nice.
4 So on top is a binding that we see in our
5 awake rats, and below is the example I showed you
6 before of the human binding with diprenorphine. And
7 we see that it's very, very similar across the brain
8 area. So now we're going on to the competitive
9 binding with an acute stimulus and then with chronic
10 pain to see if, in fact, we can alter this.
11 Then it shows you the regions where it can be
12 altered, where then you can go back and do
13 histological studies to see what's happening, to see
14 if, in fact, it's due to down regulation of the
15 receptors or whatever. But this gives you a whole
16 brain overview that then allows you to access these
17 things.
18 So quickly here, the main pitfalls in making
19 these translations is that, first of all, anesthesia
20 can suppress nociceptive responses. And this is what
21 we did with Scott, where I showed you before that
22 with this formalin, when the animals were awake, with

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1 formalin, you get a nice response in S1 hind limb.
2 We then imaged animals that had SNI injury for
3 several months, and we found that even though they
4 looked perfectly normal, we had an increase in the
5 activity in S1 cortex in these animals, suggesting
6 that there was something going on in their brains
7 related to their chronic pain.
8 This was without an acute stimulus being
9 applied. But when we did the same study with
10 isoflurane, we saw nothing. The baseline went down,
11 and there was nothing. There was no difference
12 between the SNI and the shams.
13 So I think that that anesthesia can have a
14 big effect when you have a more subtle manipulation.
15 Electric shock, okay, but not with something like
16 looking at possible ongoing activity in a chronic
17 pain model.
18 I'm not trying to bash David Borsook, but I
19 just want to show you their very first awake rat BOLD
20 heat pain study, they had 72 activations, significant
21 activations in the brain, whereas with human studies,
22 you see 5 or 6 or 10.

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1 These guys, they conditioned them. They
2 habituated them for several days. They were
3 measuring heart rate, and they found that the heat
4 pain stimulus didn't alter their heart rate. But
5 their heart rate was quite high at baseline, and
6 we've now gone back and taken animals and exposed
7 them to the same restraint.
8 I don't have the data now because I couldn't
9 track down my postdoc, who seemed to have disappeared
10 on me yesterday -- but measuring cortisol levels.
11 And these guys, their cortisol is through the roof
12 after the three days of conditioning.
13 So I think we need to do a lot more of
14 measuring what's going on because there seems to be a
15 lot more going on in these rats' brains than just
16 what you'd expect from a little heat pain stimulus.
17 So these are just things that we all have to try to
18 deal with one way or another.
19 So it seems the functional anatomical brain
20 imaging techniques developed in humans are now being
21 successfully applied to rats and mice. And these
22 techniques, I think, can allow us to examine

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1 mechanisms of clinical observations, provide whole
2 brain analysis of altered circuitry in genetically
3 modified animals, and lead to better translation
4 between preclinical and clinical models. But we must
5 remain aware of the effects of anesthesia and
6 experimental stress in interpreting the rodent
7 imaging data.
8 These are some of my main colleagues in the
9 studies that I talked about from our lab. Thank you.
10 (Applause.)
11 DR. PORRECA: Since we only have two speakers
12 in this session, we thought we'd go ahead and have
13 both presentations given, and then spend the rest of
14 the time with the open question and answer.
15 So it's my pleasure to introduce our next
16 speaker, Dr. Yves De Koninck, who is in Laval running
17 an extremely innovative and creative pain research
18 center. And Yves is going to speak about the
19 potential that's associated with the emerging
20 application of optogenetics to understanding pain
21 mechanisms.
22 So you see Yves' title here, so thanks, Yves.

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1 Presentation – Yves De Koninck
2 DR. DE KONINCK: Thanks, Frank.
3 As Frank said, I guess my job here is to try
4 to give you a feel for why I think that optogenetics
5 or optical approaches are going to be hopefully very
6 useful to bridge in what's been missing and what's
7 we've been discussing.
8 Catherine gave a great talk to really show
9 how trying to use the same modality in humans and
10 animals can be quite instrumental.
11 Another place where I think we need to bridge
12 is between cellular activity and behavior. So pain
13 is a complex behavior. Frank is always bashing this
14 on us and saying we have to be assessing pain as a
15 more complex behavior than just a reflex. But then
16 we are interested in targets that are working at the
17 cellular level, enzymatic level, biophysical level,
18 and we want to try to bridge between them.
19 I think this is where optical approaches and
20 optogenetics, in the broad sense, I think is going to
21 be quite instrumental as allowing us to do cell
22 biology, but in vivo, in context-sensitive

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1 situations, context-relevant situation.
2 I guess as disclosure, I just wanted to
3 mention that I am actually -- we have a license
4 agreement with Hoffman-La Roche on something that has
5 absolutely nothing to do with today's talk, on our
6 compounds, on chloride transport modulators. But I
7 thought I'd mention it because along with that, we
8 actually have a collaborative grant with them as
9 well.
10 Again, that's big PhRMA coming to us because
11 they are interested in these cellular imaging
12 approaches in vivo to push further their target
13 discovery and validation approaches. So it really is
14 another example of where my speech today is maybe
15 relevant to drug discovery.
16 So ultimately we want to figure out how this
17 whole thing works. The bridging part that's perhaps
18 missing in what Catherine's talk was before is that
19 the imaging approaches that she's talked about are
20 great in trying to get different regions of the brain
21 working away, trying to connect them. At some point,
22 as I said, you need to try to go down to single cells

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1 and try to decipher how the microcircuits work, how
2 the information is encoded there, and how cellular
3 processes and even molecular processes occur in these
4 small networks within the functioning brain. And
5 that's where, again, we think that optical approaches
6 are a really good translational tool for that.
7 What I like to always stress here is that I
8 take optogenetics in its broad sense where it's
9 exploiting genetically encoded proteins that respond
10 to light so that you can exploit them to both observe
11 and control. And the two things are very, very, very
12 interdependent and equally important, I think.
13 There's a lot of hype. Optogenetics, we can
14 certainly give the credit to Karl Deisseroth for
15 coining the term, and Karl really certainly pushes a
16 lot of the control aspect. And that's where there's
17 a lot of hype for that and a lot of hopes and
18 rightfully so. But I think sensing is equally
19 important. And hopefully, I'll be showing you some
20 examples of that; so both actuators and sensors to be
21 able to control the system but also observe down to
22 single cells, down to cellular process, inside cells,

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1 in the in vivo context.
2 So in my mind, the first optogenetic
3 protein -- optogenetics goes back to green
4 fluorescent protein -- any protein that is
5 photoresponsive. And then you exploit that. And
6 there's been obviously enormous work, especially from
7 Roger Chan's lab, to turn these photoresponsive
8 proteins into incredible sensors.
9 So the context is we want to figure out -- we
10 still today want to figure out today how
11 somatosensory input is encoded. And taking this
12 slide from a review by Steve Prescott, who's bringing
13 up again the concept that we have to think of the
14 system in terms of combinatorics with a combinatorics
15 view.
16 Even Allan had a review in Neuron recently
17 talking back about the gate control theory, and
18 reflecting 50 years later, we're still debating how
19 the signals are processed in the spinal cord. And
20 certainly, there are labeled lines, but everyone, I
21 think, would agree that there are interactions
22 between them and complex interactions. And we need

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1 to figure out these interactions are.
2 We'd like to think about them -- I mean, the
3 problem poses itself already at the spinal level, but
4 it recurs at every point in the relay, at the spinal
5 cord level. We can transpose the same thing to
6 higher brain structures, all the way to the cortical
7 areas.
8 So there's complex processing, multiple cells
9 interacting. We need to decode that. Optical
10 approach or optogenetic approaches offer some
11 advantage over classical electrophysiology approaches
12 in that they can bridge the genetics. We can harness
13 the genetics to do that, to monitor the different
14 players in these networks and also control the
15 different players in these networks. And hopefully,
16 as the technology progress, we can complexify this so
17 that we can monitor multiple, multiple players
18 together and control them as well.
19 So the optogenetics toolbox keeps growing
20 very fast. Perhaps it's frustrating for some of you
21 because the hopes and the promises are great, and
22 you're saying, well, how come it's not really

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1 translating. There's still quite a lot of debugging
2 that is occurring in the field. It's moving very
3 fast, and the number of Nature, whatnot, papers on
4 this is creeping up because of the expectations are
5 very high. But I think one thing to keep in mind is
6 that a lot of these -- there are still a number of
7 hurdles in building these tools.
8 But certainly, you have a bunch of tools, and
9 it will allow you to control things, to be able to
10 identify cells, monitor them and whatnot; also, using
11 these genetically encoded tools to use tracing
12 techniques and mixing these tracing techniques with
13 optogenetic tools and so on, a number of tools to try
14 to decipher the network.
15 So one example, let's start with the
16 beginning, just how the signal is encoded from the
17 periphery to the central nervous system. We have
18 these classes of -- this massive class of sensory
19 fibers. We'd like to be able to dissect out what
20 each of these are doing. Again, I was talking about
21 hurdles.
22 It takes a while before we could really get

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1 these tools right to work even in something simple as
2 a sensory system. In the last year to two, there's
3 been some good breakthroughs.
4 Here's one example that I'm illustrating
5 where we can actually use these certain viruses.
6 This was pioneered by a few groups, including David
7 Anderson's group, and finding that when you inject
8 certain viruses in the newborn, in the periphery, you
9 can actually selectively transduce sensory fibers,
10 and just sensory fibers. It's quite nice.
11 So you can then play with whatever, your
12 favorite mouse, here, an Na(v)1.8-Cre for the proof
13 of concept here, and have a virus that will encode
14 channelrhodopsin, which when activated by light will
15 excite the cell so that you can probably use light to
16 selectively activate sensory neurons.
17 So here's this Na(v)1.8 mouse, so you can see
18 just a subset of the cells are transduced here. You
19 can even see the channelrhodopsin. The reason why
20 you see is that the protein channelrhodopsin is
21 coupled to YFP, so you can actually see it in the
22 nervous system. This is the periphery. The sensory

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1 neurons are expressing it.
2 This strategy was necessary to get
3 enough -- just simple bug, just get enough
4 channelrhodopsin expressed in the afferents so that
5 you could envision being able to activate the
6 afferents with light through the skin.
7 So here's an example. Here's one mouse out
8 of these four that has it. I hope you saw which one
9 that was. The other ones don't really bother. So
10 basically, you're able to just with light -- see,
11 that little mouse is reacting to light, so we're able
12 to activate those sensory fibers with light.
13 So why is that particularly interesting?
14 This is again just proof of concept. It's just using
15 the Na(v)1.8-Cre mouse. But one place where it's
16 already bridging is to bridge between this and this;
17 the idea that now you can have an excised preparation
18 and apply the same stimulus, the same parameters and
19 whatnot, the same class of afferents selectively, and
20 study in the cellular process, for example, your
21 synaptic plasticity, an example of long-term
22 potentiation in the spinal cord that you trigger by

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1 activating these afferents.
2 Then you can do the same in the behaving
3 animal to try to produce sensitization through the
4 skin. And here is an example. When you're doing
5 withdrawal reflex to mechanical stimulation before
6 and after, you've conditioned the animal with
7 stimulating the nociceptors at a high frequency. And
8 you can see that you can produce that sensitization.
9 So, of course, the promise is then you can
10 start activating selectively each of your favorite
11 afferent and look for the responses that is evoked
12 there. There's an example. And, of course, by doing
13 this presumably you'll be producing a cleaner
14 response as you see here with this light as opposed
15 to electrical stimulation where you have a more
16 complex response here.
17 So the idea would be to try to dissect out
18 how activation of each of these afferents both in
19 vitro and in vivo will be happening.
20 Now, challenge again, high expectation but
21 challenges again, the genetic targeting and the
22 genetic identification of each of our favorite cell

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1 types still remains a challenge. I think -- and here
2 I'm illustrating one of the strategies that Quifu Ma
3 here is highlighting, and he's been pioneering with
4 Martin Goulding at UCSD, trying to do combinations.
5 I think it's going to -- I'm a strong
6 believer that if we want to start targeting our
7 favorite cells, we'll have to start doing
8 combinations of expression of different
9 factors -- here's one strategy using these two gene
10 activators, if you want -- but also combining genetic
11 targeting with different promoters and tracing
12 techniques and whatnot. We'll have to be able to
13 play with all of these things to really narrow down
14 on the selective subsets of cells that we're
15 interested in.
16 So the beauty of this, of course, is that if
17 you have just the cell that you're interested in
18 express the light sensitive channels or light
19 sensitive whatever you're interested in, it's nice
20 because then you can just do bulk light excitation.
21 Here it is illustrated with just a fiber optic stuck
22 in the head of the animal. And then you will

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1 hopefully be able to just target, activate, just the
2 cells that you're interested in.
3 The same logic is true for sensing. By
4 targeting sensors, so protein sensors that will be
5 sensing activity, enzymatic activity, neuronal
6 activity or whatnot, neuronal morphology and whatnot,
7 you can really follow that in vivo.
8 So to illustrate that, just a few examples.
9 Again, challenges, we need to develop tools to be
10 able to follow these small networks. Here's an
11 example of fancy microscopes that we're designing in
12 our group to try to be able to follow the networks of
13 cells in sensory ganglia and in dorsal horn.
14 One of the simple issues that you face when
15 you develop these things, just to illustrate the
16 hurdles that are there, is that spinal cord tissue
17 moves big time. Here's an example of stabilization
18 process, which you saw systems stabilizing when you
19 turn it on, based on a feedback system that actually
20 senses the surface of the tissue and feeds back on
21 the objective.
22 So what we're actually seeing there is a

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1 stable image. The tissue is not stable. The image
2 is stable because the microscope follows in real
3 time.
4 So then the promise of this, of course, these
5 imaging approaches is that to record from a number of
6 cells at the same time and see how they can encode
7 the information.
8 So let's step back again to our sensory
9 fibers, sort of maybe plead for the opposite of what
10 I said before. I said that with the genetic
11 targeting, one nice thing is that we'll be able to
12 dissect out how each component works. But it's also
13 interesting to try to monitor how the whole ensemble
14 is dancing and dancing around.
15 So here again using our viral strategy to
16 transduce selectively sensory neurons, here we're
17 putting in the neurons A sensor to GCaMP6, calcium
18 sensing protein, which will be a proxy of activity of
19 the cells. Again, the field is moving now very fast
20 thanks to this game changer GCaMP6. Before that, a
21 lot of iterations before that, a lot of hurdles, a
22 lot of limitations, and then especially, translation

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1 in using them as tools for in vivo.
2 So again, as I told you, the beauty of this
3 AAV9 is that if you inject that in the periphery, you
4 really infect -- transduce -- the vast majority of
5 the sensory fibers just in the DRG, like you see
6 this. You see that in the dorsal horn. You see just
7 the sensory fibers entering, but there's no dorsal
8 horn neuron that were transduced by that. And you
9 can really label the vast majority of the DRG neurons
10 here just going through the ganglia here to see all
11 cells that are labeled with GCaMP.
12 So that you can just then monitor an ensemble
13 of neuron responding to a sensory input. Here's an
14 example of the cells that are just responding to a
15 noxious heat to the foot, and we're recording in the
16 DRG.
17 The point I want to make here with this
18 slide, just to provoke your thoughts, is this is just
19 a early mapping, crude mapping, of the array of
20 responses that you have in the DRG to your stimuli.
21 So you have cells -- you're getting cold stimuli,
22 warm stimuli, heat stimuli. You have a few cells

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1 that are responding just to heat, a few that are
2 responding just to cold, some to warm, but you have a
3 whole array of cells in the gray zone here that are
4 responding, to some degree, to all of these
5 modalities.
6 So that in the end when you give a
7 stimulus -- so you have cells. We know we have cells
8 that are tuned optimally to a stimulus. But when you
9 give a stimulus in real life, stimulus is always
10 acting in a number of different cells. Each cell has
11 a spectrum of activation, so that you'll be
12 activating an array of cells. And, in fact, I would
13 push even further that in many stimuli, never occur
14 in isolation. I can't pinch you without touching you
15 before. So the fact that these two stimuli are
16 occurring often concurrently probably means something
17 to our neuro system.
18 So the ultimate, I think, experiment will be
19 to decode or to figure out how all of this
20 information is encoded, something as early as the
21 DRG, and then play it back in the system and see how
22 the system is going to behave. And what I'm

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1 illustrating here in the DRG, you can transport each
2 level in the CNS, spinal cord, brain and so on. Each
3 of these places, information is going to be encoded,
4 translated, sent further down to be encoded further.
5 So actually to link to what Catherine was
6 saying before, this is great. You saw the heavy
7 microscope. I have to anesthetize the animal in that
8 case. Anesthesia could be an issue for sensory
9 neurons, primary sensory neurons, perhaps not too
10 much, but then as you go further down the brain, down
11 the neuraxis, it's going to become more critical. So
12 obviously, you want to develop approaches to try to
13 follow this in vivo. Again, a lot of development in
14 the works.
15 Here's an example of a spinal window that you
16 can use that we've been developing and others have
17 been developing to try to follow things at the spinal
18 level. Again, spinal just being more challenging
19 than the rest of the higher up in the brain. You can
20 see that these little mice move happily with this
21 little window.
22 There were inflammation issues. This has

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1 been a lot debugged and worked out. Here I reference
2 work by Franck Debarbieux, who's done a lot of great
3 work at re-optimizing the spinal windows to minimize
4 any inflammatory responses.
5 What I want to illustrate here is
6 that -- well, so this window perhaps is a bit
7 invasive. Another approach that is being explored a
8 lot in the field and developed is to try to develop
9 these small micro-endoscopes to try to have the most
10 minimal intrusion into the brain for the spinal cord.
11 This turns out to be great because, in fact, even in
12 little mice, you can insert these micro-endoscopes in
13 between vertebrae without having to break any of the
14 bones. And you can do the imaging across the dura,
15 so we can do relatively noninvasive imaging.
16 So what I showed you before is a lot of
17 imaging using GCaMPs, calcium imaging as a proxy of
18 neuronal activity. There are a lot of other cells in
19 CNS. Here's just an example of another cell that we
20 like or hate, whatever, the little microglial cells.
21 They don't have the same type of electrical activity
22 that neurons have, but they are very active. You

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1 need to be able to follow how these cells are
2 behaving in tissue.
3 So here's an example of where again imaging,
4 in situ, through the optogenetic approach so that you
5 can just see those cells can be quite instrumental.
6 Here's a mouse that expresses GFP inside microglial
7 cells. You see the little cells are just scanning.
8 That's what these cells do. That's one of their
9 behaviors that's critical, is that they move around
10 scanning the environment, and they respond to injury.
11 So what you can do is you can come in with a
12 laser and just make a little hole in the middle to
13 see how these cells will react to that hole, and you
14 see that they do come in and actually are
15 circumscribing the area to protect that damaged area.
16 This is done through the dura without going into the
17 tissue.
18 Now, the reason I'm showing this again is to
19 say that the physical behavior of these cells
20 characterizes them, and you can actually quantify
21 that. Just to illustrate my point here, for example,
22 when you do a nerve injury and you're measuring in

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1 the spinal cord, not the area that you've damaged,
2 you can see that the cells decrease a lot their
3 mobility. They become activated and whatnot, fat.
4 They just don't move. But when you actually do a
5 lesion, they actually respond faster. So something
6 has happened that is priming these cells, some
7 signaling mechanisms are ongoing there, and you can
8 actually see that in the dynamic behavior of the
9 cells.
10 Just again to illustrate that, seeing things
11 in action through these approaches can reveal things
12 that you wouldn't be able to see in static
13 approaches, for example.
14 Now, you want to go higher up in the brain.
15 That's the area that Frank only cares about now. And
16 certainly, ultimately, we want to go and see how is
17 information encoded higher up there. Of course,
18 there are several techniques to monitor cortical
19 activities, and Catherine has shown great stuff that
20 you can do with imaging.
21 What optical approaches offer perhaps over
22 other approaches is very fast time scale; again,

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1 trying to figure out how the brain encodes
2 information needs fast time scale monitoring.
3 Here's an example from work from Tim Murphy's
4 group who's actually using voltage sensitive dyes.
5 This is a dye in this case, though it's not
6 optogenetics. But it really shows that you can
7 really follow across levels cortical activity, and
8 then you can -- just doing the same type of
9 correlative activity that the MRI community is doing
10 now to look at functional connectivity between
11 different areas. You can do this at a smaller
12 spatial scale but much faster temporal scale to try
13 to figure out how the connectivity is organized
14 there.
15 You could argue that this is surface
16 recording in the cortex. That's one of the problems
17 with light is you record very much at the surface.
18 Tissue is very scattering. Brain tissue is very
19 scattering, so you can't really go deep into the
20 tissue to figure out how things are working there.
21 So you could argue, well, maybe electrical approaches
22 or EEGs have been telling us quite a bit on the

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1 cortical surface activity. And if we want to be able
2 to really decipher, especially in the pain system,
3 how things are happening, you want to be able to go
4 deeper into the tissue.
5 So that's where, again, these little
6 micro-endoscope approach that I told you about are
7 quite instrumental, something that's been pioneered
8 by Mark Schnitzer at Stanford. Here you have one of
9 his little favorite mini, mini microscope, small
10 enough that a little mouse can walk with it on the
11 head, and you can insert the little micro-endoscope
12 in the head.
13 The objective here is to monitor cellular
14 activity in the network. Here you have it inserted
15 in the hippocampus. This is actually a mouse moving
16 around and looking at place cells in the hippocampus.
17 And you can see how this mouse responds, how the
18 network responds to a specific context and whatnot.
19 The objective ultimately is to again decode how all
20 of this information is happening.
21 Sometimes what you want, though -- or some of
22 the cells that you're interested in are very rare and

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1 sparsely distributed. Some of us are obsessed with
2 some little cell types that are very rare. This is
3 an example of cells that we're interested in, the
4 little cholinergic neurons in the dorsal horn of the
5 spinal cord. I just used that as an example. You
6 have tons of cholinergic neurons in the ventral horn,
7 but in the dorsal horn is actually forming a very,
8 very small subpopulation of cells.

9 So you need to be able to actually go in, in
10 context again, and follow how each of these cells are
11 behaving inside the intact animal. And even though
12 some of them may be scarcely distributed, they
13 sometimes have very important impact on overall
14 network activity, like, for example, these
15 cholinergic interneurons.

16 So one way to get at that that we developed
17 is a little microprobe that's basically making a
18 glass micro-electrode that we've been using for ages
19 to record electrical activity, but making it through
20 a fiber optic. So it's actually using an all glass
21 strategy, fiber optic that has an optical core. So
22 fiber optic is just glass inside glass with an

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1 interface to guide the light, but then we also add a
2 little hollow tube in the perform.

3 So then you can have your glass probe that
4 you can pull down. You have the hollow core that you
5 can fill with an electrolyte, and then have
6 electrical contact to the tip as well as optical
7 contact in the tip. So the idea is then to go down
8 and be able to use both electrical activity to study
9 your favorite cell, but also of an optical channel to
10 see what's happening there.

11 So use light again to probe beyond just
12 electrical activity, for example, in the cell. So
13 the beauty of that is if you can inject the light and
14 collect the light through the same fibers, so you can
15 go down to your bottom, if you want.

16 So the idea is you just go down. You scan
17 down, and as you're getting close to the cell, the
18 light will go up and down so you can identify your
19 cell. This is an example where we've actually used
20 this in a mouse that had green and red cells. This
21 is in the striatum, the D1 and D2 cells.

22 You can see that when you get close to a

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1 green cell, you see the cell as you're passing by, or
2 a red cell, you see it as you're passing by. We get
3 the same proportion of cells with either just looking
4 at the tissue or sampling them with our probes. So
5 that's making us confident that we can sample the
6 different types of cells.

7 So you can envisage then going in vivo,
8 studying your favorite cell and how it responds to
9 different interventions. Think of drug intervention,
10 for example. Understanding how the cell that you're
11 interested in, the sub-cell that you're interested
12 in, will respond to drug application, but in an
13 intact brain. And ultimately, you'd want to do that
14 in a behaving animal. We'll get to that.

15 Of course, if you have a probe like that, you
16 can try to play with it and do optogenetics. Well,
17 optogenetics -- see, I'm using the same
18 bias -- optical activation.

19 This is a transgenic mouse where you have
20 channelrhodopsin distributed in different areas. You
21 can go down next to cells that you're interested in,
22 and then you can flash light. And you can see that

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1 your cell can respond to your light. See the little
2 thing here; this is action potentials of the cells as
3 you're shining light on it.

4 Now, this is superficial. You can ask what
5 are the things that you can do. There are a number
6 of things that you could do now that you can
7 manipulate cells with light at the single cell level
8 deep into the tissue.

9 But I want to come back to what is actually
10 more dear to me, is monitoring, using this to monitor
11 activity. This is an example taken from the
12 thalamus. You go down to thalamic cells that respond
13 to whiskers. You can move the whiskers around.
14 These thalamic cells now have calcium indicators in
15 them. So you can see that when you move the whisker,
16 you get an action potential, and you get a calcium
17 response.

18 You see it better here. The cell is
19 bursting. You see nice calcium response, single
20 traces, very nice signal to noise ratio.

21 So this is telling us that now we can use
22 light to actually monitor in vivo deep in the brain

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1 individual cell activity or individual signaling
2 events. Here this is just an ionic mechanism. You
3 could imagine an enzymatic mechanism, whatnot.
4 Now, just to close, this is going full circle
5 to what perhaps what's dear to Frank at least. Frank
6 would be telling you, well, if you want to assess
7 pain, you have to assess what goes on here in the
8 brain, and there's a whole bunch of nuclei there that
9 are important, reward pathways, whatnot.
10 So what Frank is using as a strategy is
11 saying, well, I'm going to be assessing this using
12 behavioral assays, and they're going to be my
13 endpoints or my proxies of how these pathways are
14 acting. And they are reflecting, if you want, the
15 state of the animal, closer proxy to actual pain than
16 these reflex mechanisms that we've been studying.
17 So my sort of dream project through this is
18 to try to add, go further in this direction in
19 saying, well, okay, you can study this in behavior.
20 But it would be nice if we had ways of actually
21 monitoring in these different areas activity as the
22 animal is just walking around and doing things.

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1 One of the ways that you can do that is to
2 pick up on that little microprobe that I told you
3 about and go into the different little brain areas
4 that you're interested in. And the reason why I
5 think our approach may be a little more translatable
6 towards that is because the idea is you want to have
7 an animal that has a few needles in its head that are
8 just monitoring activity.
9 So you could say, well, people have been
10 doing this with electrophysiology over the years.
11 Well, sure, electrophysiology will -- you can do
12 chronic electrophysiology in animals. It won't give
13 you easily information on the molecular identities of
14 the cells that you're interested in. And, of course,
15 it won't allow you to control these cells.
16 Again, what the optogenetic approach is
17 offering to us is a way of targeting the different
18 cells that you're interested in and multiplexing the
19 information. So here is actually exploiting, for
20 example, the recent developments on the GCaMPs, on
21 the calcium sensitive fluorescent proteins. And
22 Robert Campbell in Canada has made green, blue, red

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1 variants of that. So now you can have multiple
2 signals that you could target to different
3 compartments.
4 So the difference here, I showed you before
5 Mark Schnitzer's micro-endoscope who can record,
6 image the network. You can do imaging. It's fairly
7 invasive, though, because you have to image a fairly
8 large area, bulky thing. This little microelectrode
9 that I told you is not an imaging device. With
10 fibers, you don't image. It's a point measurement.
11 So you actually don't have spatial information. But
12 specific spatial if you want information, you can try
13 to regain it by using colors.
14 So here's an example from Robert's
15 collaboration with Nagai's group. Here they've
16 actually targeted the different colors to different
17 cellular compartments, so nucleus, endoplasmic
18 reticulum, cytoplasm. And you can see that the cells
19 are responding. So each of these are signaling
20 calcium, but each in their own respective
21 compartments.
22 If you forget the spatial information that

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1 you have there in the image and you just look at it
2 temporally, you see that calcium oscillations are
3 following different kinetics in each of these
4 compartments.
5 So if I'm interested in calcium in the
6 endoplasmic reticulum, I just need to target
7 something there and just study what happens in the
8 endoplasmic reticulum. I don't necessarily need to
9 actually see the endoplasmic reticulum.
10 So the same concept you can transpose in the
11 brain area, the your favorite brain nucleus that you
12 care about, that has five, whatever, three through
13 two, seven cell types that you can target with
14 different colors.
15 So then you can envisage that you can bring
16 your probe down, and the probe can be able to be
17 specific to one color, for example. So only the
18 cells that are blue, for example, are going to be
19 firing away. Then you can try to scale this back,
20 try to sample a larger area and do multicolor
21 sampling or even further sampling.
22 So the idea is to envisage having a small

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1 probe in one area and sample activity without spatial
2 information in the sense of imaging but monitoring
3 activity from X, Y and Z cell type. And that, I
4 think, is going to be step forward from, for example,
5 one of the criticisms that we often have with
6 conventional imaging approaches with MRI or
7 functional MRI is that you don't necessarily know
8 which cells are actually firing during this or that
9 behavior.

10 All right. So this is just my usual
11 advertisement for those who are interested or have
12 students. We run the summer school. What I told you
13 about today is actually happening in a center that I
14 founded, which is actually a whole platform that's an
15 interface between physicists and biologists. It's
16 all aimed at developing new techniques.

17 So there's a huge work force that are working
18 on trying to improve the hardware to do the kinds of
19 things that I'm telling you, and we run a summer
20 school every year on many of these advanced
21 techniques. Et voila.

22 (Applause.)

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1 Q&A – Bushnell and Koninck

2 DR. BUSHNELL: Some people are now beginning
3 to combine these things, so you stimulate specific
4 cells with optogenetics, and then you can do whole
5 brain imaging about where they're going, which is
6 cool.

7 DR. DE KONINCK: Sure, absolutely. And, in
8 fact, I think the future is going to -- the concept
9 right now, light is very amenable to this. But I
10 think there are other modalities that will emerge
11 again with the same principle of being able to
12 control very selectively.

13 DR. BASBAUM: I'm just going to let you
14 change direction for a second, and I'm surprised
15 actually, Yves, that there was no mention of DREADD
16 technology.

17 DR. DE KONINCK: Yes, okay. Good point,
18 Allan, and I guess it's just because optogenetics was
19 the title, and I just tried to focus on there.

20 Again, as we were just saying, other
21 modalities, and one of them is chemicals, so chemical
22 specificity. The optogenetics field is sort of often

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1 the most talked about out there because the promise
2 there is to have very precise temporal control of the
3 cells.

4 So the idea, the ultimate experiment in a
5 sense is to have the -- we see these networks of
6 neurons that are firing away. If you could just
7 basically tape this and play it back, it would be
8 really great to see whether this or that pattern of
9 activity in these different cells is sufficient to
10 explain this or that behavior.

11 There the temporal precision is quite
12 important, and the optical approaches are great. But
13 for certain, other interventions, which you would
14 want is to replicate like, say, for example,
15 continuous activation of a pathway. And in that
16 case, the chemical approaches might be more
17 appropriate than having to inject light continuously.

18 I assume everyone understood what Allan said
19 when he said "DREADD." So he's just talking about
20 essentially the idea is to use a non-endogenous
21 receptor that's coupled to an effect or mechanism
22 that will activate or inactivate cells. And if this

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1 receptor is responding to an agonist that is not
2 endogenous, that the animal never sees, then you can
3 use that agonist to selectively activate the cells
4 that expressed, that you've made express that
5 receptor.

6 DR. MCMAHON: So the optical approach
7 obviously is dramatic and fantastic, but there must
8 be some snags. And you didn't tell us about any of
9 the disadvantages.

10 DR. DE KONINCK: Yes.

11 DR. MCMAHON: So in the spirit of
12 cooperation, things like the sensitivity of different
13 cells or sustainability of current with continuous
14 light, I mean, are there significant problems?

15 DR. DE KONINCK: Yes, and, in fact, old
16 timers like you and I, Steve, who've been doing
17 electrophysiology for years, are seeing that the
18 whole field of optogenetics is just rediscovering
19 electrophysiology. And they're rediscovering a
20 number of artifacts of electrophysiology. And there
21 are definitely a number of artifacts.

22 One thing that you mentioned is the fact

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1 that, for example, I guess in your field with sensory
2 fibers that are transducers, they are transducing a
3 physical stimulus that has a certain physical flavor.
4 With light, when you are activating the afferent with
5 light, you're not necessarily replicating that. So
6 there's something that may not be physiological
7 there.
8 Another bigger aspect that's -- well, again,
9 I was mentioning that there's a lot of debugging in
10 the field. Channelrhodopsin actually turns out to
11 have been the tool that won the battle because it's
12 all genetically encoded single channel. You don't
13 have to add extraneous agents to make it light
14 responsive.
15 But channelrhodopsin turns out to be one of
16 the worst of all. Its conductance is awful. And the
17 properties of its conductance are really awful. So
18 the unit conductance is very small, so you need tons
19 of it. That's one of the problems.
20 That's why, for example, when Mark Zylka
21 tried to make his first light activated MrgD
22 mouse -- so he had this subclass of afferents, and he

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1 just said I'll just make it transgenic and be able to
2 activate these subclass of afferents -- it worked
3 fine, and the DRG worked fine in the spinal cord.
4 But through the skin didn't work. Basically the idea
5 was that there was not enough channelrhodopsin.
6 So you need a lot of protein. If you need a
7 lot of protein, you're a little worried. That could
8 be a problem for the cells.
9 A lot of work out there, tremendous amount of
10 work in improving the conductance, kinetics,
11 properties of this ion channels. So that's improving
12 over time.
13 One thing that is emerging, for example, in
14 people that are studying sense synaptic physiology is
15 that we used to do it nicely with stimulating
16 electrodes, and we'd get responses. What you do
17 there is you generate an action potential, let's say
18 in the axon, and it's translating down to the
19 terminals. Some people are trying to use optical
20 approaches to stimulate directly the terminals.
21 But when you do that, again, you're doing it
22 in a very different way than an invading action

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1 potential. So people see papers emerging now that
2 are showing that, well, the plasticity properties are
3 not necessarily exactly the same when you do it like
4 that. So there's a number of things that people have
5 to be worried about, the usual with every new
6 technique that's coming out.
7 DR. PORRECA: Yves, you mentioned a couple of
8 things that I found very striking. The difference
9 between the activation of the afferents with light as
10 opposed to the electrical stimulus, where you get
11 the --
12 DR. DE KONINCK: No, to natural stimulus I
13 meant. Oh, well, okay. Go ahead.
14 DR. PORRECA: You were pointing that you
15 could be very, very selective in which fiber class
16 you activate, and that that's not the normal way in
17 which the nervous system sees the afferent input. So
18 you're deconstructing the signal into its component
19 parts, but how does --
20 DR. DE KONINCK: But eventually you want to
21 reconstruct it, yes.
22 DR. PORRECA: Yes, exactly.

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1 DR. DE KONINCK: Absolutely. So I think the
2 two approaches are very complementary, and that's why
3 I wanted to show them like that; that it will be
4 interesting to dissect things out, but we have to
5 understand again that these things are very
6 artificial.
7 That's why eventually we want to bring
8 everything back in vivo. It's the same with all of
9 our approaches, our reduction to these approaches.
10 We like to isolate components, but you need to
11 actually be able to look at them back in the
12 functional context.
13 DR. PORRECA: And to understand what the
14 meaning is to the brain if the brain has never seen
15 that isolated signal.
16 DR. DE KONINCK: Absolutely.
17 DR. PORRECA: So the other question is
18 that -- since we were talking --
19 DR. DE KONINCK: But that may be what pain
20 is, you know, input from some afferents when others
21 are not coming in.
22 DR. PORRECA: Well, maybe. So related to

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1 this, I think is the question of since the
2 aversiveness of pain is highly dependent on stimulus
3 intensity, how do you control the stimulus intensity
4 in a way that you can modulate in a graded fashion,
5 for example? How do you really get a finer control
6 of --
7 DR. DE KONINCK: Well, I guess it
8 depends -- can see it at the single cell level. So
9 if the cell is going to be responding to one spike or
10 several spikes or a burst of spiking, whatnot, to a
11 given stimulus, or as an ensemble, whether 1 or 2 or
12 12 cells are going to be firing to your stimulus.
13 I think you can replicate that, to a certain
14 extent, with light. It has its limitation, but you
15 can certainly control duration, frequencies with
16 light. Again, that's where the temporal
17 aspect -- the fact that you can control very fast
18 cells has promises, where you try to replicate the
19 same type of strains that you see in an afferent.
20 But again, threshold may be an issue.
21 DR. BASBAUM: You can all leave if you want.
22 We're just going to keep asking questions.

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1 (Laughter.)
2 DR. BASBAUM: I have a kind of general
3 question for everybody, and I wanted to ask it
4 before. We talk about -- and there was an
5 interesting discussion about TRPV1, and just now we
6 talked about using heat or light to activate. You
7 showed subpopulation of dorsal root ganglion cells.
8 And, Catherine, you will often use heat as a stimulus
9 certainly in humans.
10 I've always thought of heat, in engaging
11 TRPV1 afferents, for example, as really a surrogate
12 for some stimulus that produces pain in humans.
13 Patients don't -- they might come in with nerve
14 injury and complain about burning pain, but they
15 rarely come in complaining that, well, gee, I have
16 arthritis and my hand is hot. No, they're
17 sensitized. It hurts when they move. There's
18 allodynia, even though the TRPV1 afferents might be
19 engaged under those conditions.
20 So I see heat as really a surrogate that
21 we're using to study something that is happening
22 clinically.

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1 How do you translate -- can you translate in
2 the animal, by imaging other patients or even in an
3 mouse model in the brain, a heat stimulus to the
4 injury stimulus? Is the heat stimulus too specific
5 to really understand what's going on? In other
6 words, in the injury condition, does that population
7 ever really get stimulated independently? It's kind
8 of the question that I think Frank was getting at.
9 Do you see where I'm going? I don't believe
10 patients come in and say, "Oh, my TRPV1 afferents
11 really got activated badly last night."
12 DR. BUSHNELL: That might be one reason why
13 taking this to the clinic doesn't work very well.
14 DR. DE KONINCK: But I think that's why I was
15 showing that slide at the DRG, where you're getting a
16 natural stimulus, and the stimulus itself is actually
17 a very heterogeneous stimulus. It takes time before
18 you reach noxious heat. You're activating a whole
19 bunch of afferents before. There are a wide array of
20 afferents that are responding to that stimulus.
21 Again, I think being able to do monitoring of
22 multiple cells at the same time, and that's one of

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1 the promises of the optical approaches over
2 electrophysiology. In fact, we've done enormous
3 amount of work in the sensory neurons characterizing
4 individual cells, their dynamic response to stimulus
5 and so on because with electrophysiology, we could
6 isolate each of them.
7 I think, indeed, we have to go back into the
8 more complex thing. And the same that I showed at
9 the DRG level is true at each level. And that's why
10 I think Catherine is saying we need to basically use
11 these approaches to see what matrix of activation
12 occurs in the brain to that stimulus.
13 DR. BUSHNELL: I mean, being able to
14 selectively monitor certain cell types and then
15 taking it out to the chronic pain condition and
16 seeing what's happening to the very stimuli, that we
17 know what happens acutely. But knowing specifically
18 the different cell types, I mean -- which is the
19 beauty of that, being able to --
20 DR. DE KONINCK: Yes, and Vanya is not there
21 to see it, so I'll see it. And of course, we can't
22 just do this in control animals. We have to do this

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1 in models of chronic pain.
2 DR. PORRECA: Right, and so that was where I
3 was just going to pull up and ask Catherine if you
4 could comment on whether or not the imaging in
5 animals has progressed to the point where we can
6 start to look at the effects of drugs in chronic pain
7 situations, or even if not in chronic pain situations
8 but at least in sensitized states.
9 Can you see differential effects of drugs
10 with the animal imaging, as I think has been done in
11 a couple of examples of human imaging?
12 DR. BUSHNELL: Yes, I mean, I think imaging
13 chronic pain -- so we have one study where we show
14 cortical activation in SNI rats that are just walking
15 around looking normal, and whether this is evoked or
16 spontaneous, but at least you're showing differential
17 cortical activation.
18 Although we saw that in the sensory areas, we
19 didn't see it in the cingulate cortex in the more
20 affective areas. And I don't know if that's because
21 they really don't have the same affect as with acute
22 pain. But I think by being able to do a whole brain

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1 imaging where you can specifically look at chronic
2 pain activation -- but this requires either -- this
3 development of the arterial spin labeling will allow
4 us to be able to do that much better with the whole
5 BOLD technique that is not amenable to looking at
6 chronic pain.
7 So either using PET imaging for metabolic
8 activity, which is more complicated, or using
9 arterial spin labeling with MRI will allow us to be
10 able to look at activations related to chronic pain,
11 which would be a lot easier to --
12 DR. PORRECA: I was thinking of the study
13 that was done by Iannetti and Irene Tracey with the
14 gabapentin, showing activation and deactivation only
15 in sensitized states in human subjects, and whether
16 or not we could capture that kind of activity in a
17 preclinical setting to differentiate mechanisms in
18 areas of actions of new drugs.
19 DR. DE KONINCK: And when you say chronic,
20 you mean how much time after the injury, to come back
21 to Laura's point, and Catherine? Are we talking --
22 DR. BUSHNELL Well, I was just saying that

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1 when we looked at what happened to these anatomical
2 changes in the brain that we see consistently in pain
3 patients, it took 20 weeks in a rat for that to be
4 significant. And it was interesting that -- I didn't
5 show this, but we were doing these anxiety tests and
6 things, other tests, and the animals looked perfectly
7 normal until about that same time period where they
8 started getting prefrontal changes. They started
9 developing anxiety-like behavior then, and they
10 didn't have it for the first 20 weeks after injury.
11 So I think that really these rats are not
12 looking like chronic pain patients until way out
13 there, and we have to start going --
14 DR. BOUNTRA: Thanks, Frank.
15 I mean, Catherine, I appreciate all the
16 advances that you're making in brain imaging. And,
17 Yves, what you're doing on the optogenetics is just
18 mind blowing. But of course, we can only do that in
19 animals. We can't do it in humans.
20 I can see with the optogenetics, we can ask
21 questions like sort of what cell type's involved or
22 maybe what's the pathway, and we can come up with

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1 hypotheses, et cetera. But I'm just thinking in the
2 clinical situation, what we really want to know is
3 where's the pathology. Is it the afferent nerves,
4 the cord going up or down, in the brain, where in the
5 brain, et cetera? Which treatment is most likely to
6 work?
7 Now, I don't know the literature well enough,
8 but in terms of sort of non-invasive electrical
9 measurements in humans, how are those advancing?
10 Because I think those could be very powerful in terms
11 of really trying to understand what is causing the
12 pain in a particular individual and which treatment
13 is most likely to work.
14 DR. BUSHNELL: When you say non-invasive
15 electrical stimulation, what are you referring to?
16 DR. BOUNTRA: Yes, ideally in the human, of
17 course, we want something that's non-invasive. But I
18 just -- Yves, you're the technical genius here. I
19 mean, I just don't know what advances are being made
20 there.
21 DR. BUSHNELL: Of being able to look at the
22 single unit level in the humans you're saying or --

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1 DR. BOUNTRA: No, not necessarily. We don't
2 need that. I mean, I think in the human -- at the
3 moment I sense a lot of neurologists, when patients
4 come in, they just try a whole battery of drugs one
5 by one. They'll try one anticonvulsant, and then
6 they'll try another one. And they'll try tricyclic
7 antidepressants. They have no idea in many cases
8 where the pathology is. It's a bit hit and miss.
9 They don't know which patients are going to respond.
10 Is there some way we could improve that?
11 DR. BUSHNELL: I just think the whole issue
12 of using, say, human brain imaging as a surrogate has
13 problems in that if the patient is getting pain
14 relief but you're not seeing it on a brain image,
15 well, which is right? Is it the brain image, or vice
16 versa?
17 I think that it's too crude to be able to use
18 it predictively. And again, these drugs are ones
19 that take time to develop. And so you still have to
20 do a long time frame. So not asking the patient and
21 trying to use these as a surrogate is not going to
22 get us very far. It's just not. It's too crude.

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1 I mean, there may be some instances where
2 somebody is not able to report their pain and you can
3 use it in that case as a crude surrogate, that's
4 fine. But I think if you have patient reports, I'm
5 personally -- this is my personal opinion and not all
6 people who do brain imaging agree with me. But I
7 think that it's not getting you a lot farther than
8 patient reports.
9 DR. DE KONINCK: What I'd like to add to
10 this, though, is one of the promises, and one I often
11 talk about -- I focus here on optogenetics because
12 that was the topic. But a lot of the technologies
13 that we push in our center are optical approaches
14 that allow us to do measurements in tissue that are
15 beyond just optogenetic measurement.
16 One of the promises of optical approaches is
17 molecular specificity, the fact that you can measure
18 things more directly, neurovascular response,
19 neurometabolic response, detect directly metabolic
20 proxies, even chemicals directly with light.
21 I'd like to make a link here to one of the
22 things that Steve mentioned earlier, that in the

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1 instrumentation field, new things are advancing fast.
2 And there are a lot of things that we stick in
3 people, and certainly, spinal stimulators are a great
4 example of that.
5 What I think is missing a lot in this whole
6 field is the other part of the loop, the ability to
7 actually measure what happens in the tissue, to
8 tailor your intervention. And I think I'm one that
9 believes that fiber optic technologies, which is
10 quite translatable to humans for implants like
11 microelectrodes are and whatnot in different areas,
12 are going to be very enabling in that, using them to
13 actually measure the impact of our intervention or
14 even doing early measurements to guide intervention.
15 DR. BOUNTRA: I'm sorry. I think that's
16 exactly what I was trying to get at, Yves. I was
17 just thinking if we had a room full of clinicians
18 here, what are the questions they want to know, or a
19 group full of people trying to do drug discovery,
20 what are the key questions?
21 The questions are where is the pathology,
22 which treatment is most likely to work in this

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1 individual, how long is it going to take, is this
2 compound getting across the blood-brain barrier, is
3 this compound acting peripherally or centrally, those
4 sorts of things --
5 DR. DE KONINCK: And coincidence of events,
6 so the temporal aspect is quite important. Like in
7 the epilepsy field, people are trying to detect the
8 early signs of a seizure coming in to intervene. I
9 think the same type of logic has to be used in the
10 field of pain or migraine or whatnot.
11 DR. BOUNTRA: Yes, I agree.
12 DR. PORRECA: Any other questions from the
13 audience?
14 (No response.)
15 Adjournment
16 DR. PORRECA: Well, okay. Well, thank you,
17 everybody. I think it was a very outstanding
18 session, amazing work. Thank you very much.
19 (Applause.)
20 (Whereupon, at 5:06 p.m., the workshop was
21 adjourned.)
22

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