CURE Epilepsy Seminar
Understanding the Mechanisms of Epilepsy in mTORopathies
(Transcript)

Priya Balasubramanian: 00:00 Hi, everyone. Welcome. I'm Priya Balasubramanianmanian, and I am Associate Director of Research at CURE Epilepsy. I want to thank you all for joining us today. So today we bring you our next virtual seminar of 2021 and this is part of our Frontiers in Research Seminar Series. Today's seminar is entitled "Understanding the Mechanisms of Epilepsy and in mTORopathies (FCDII and TSC)". We'll discuss the role of HCN4 as an mTOR-dependent driver of epilepsy in TSC and FCD.

Priya Balasubramanian: 00:37 Our Frontiers and Research Seminar Series is generously supported by the [Nussenbaum-Vogelstein 00:00:42] family. It aims to help educate and expose researchers, clinicians, and students to exciting epilepsy research, and also provide opportunities for young investigators to interact with leaders in the field. As a result of the pandemic and the social distancing guidelines, we have been unable to provide this interaction live at academic institutions around the world. For now until we can all come back together again, we will continue to present these seminars virtually in 2021.

Priya Balasubramanian: 01:19 As you all know, CURE has been proud to be a leader in the epilepsy research community for over 20 years. We've funded over 260 projects, spanning 16 countries. We have three different funding mechanisms right now and our key priority research areas include the acquired and pediatric epilepsies, treatment-resistant epilepsies, sleep and epilepsy, and sudden unexpected death in epilepsy or SUDEP.

Priya Balasubramanian: 01:46 All of our grant applications, they go through a letter of intent phase and also full proposal review. Full proposals are reviewed by both scientific reviewers as well as members of our community who are touched by epilepsy. Of the three, our Catalyst Award is our newest award and this is intended to fund translational research that aims to advance new therapies into clinical application. We did release the RFA for this award, and it is available on our website. We will be accepting LOIs starting June 1st. You might want to mark that date down. The CURE Epilepsy Award is open to establish investigators, whereas the Taking Flight Award is intended to support junior investigators who have at least three years of postdoc experience but have not yet obtained significant funding. The next call for these will be next year.

Priya Balasubramanian: 02:41 I'm really happy today to welcome Dr. Angelique Bordey. She is Professor of Neurosurgery and Cellular and Molecular Physiology at Yale School of Medicine. She is also a member of

the CURE Epilepsy Scientific Advisory Council, and a former grantee. Before Angelique begins, I'd like to encourage everyone to ask questions. You can submit your questions at any time during the presentation by typing them in the Q&A tab located on the bottom of your Zoom panel and click send. We'll do our best to get through as many questions as we can. I also want to mention that today's virtual seminar as well as all of our future seminars are recorded, and they are available on our website. And with that, I'll turn it over to Angelique. Thank you.

Dr. Angelique Bordey: 03:33 Well, thank you for the opportunity to give a presentation here. Hello, everybody that is invisible to me. I will try to present some of the mechanism that may contribute to seizure and epilepsy in two different types of interrelated disorder, focal cortical dysplasia type 2 and tuberous sclerosis complex, TSC. Let me just...

Dr. Angelique Bordey: 04:08 There are several of these neurodevelopmental disorders that are associated with gene variants along the mTOR signaling pathways that ultimately lead to mTOR hyperactivity and seizures in about 80% to 90% of these patients. So, different genes that define different neurodevelopmental disorder, TSC, FCD type 2 with a lot of genetic variance, hemimegalencephaly and Cowden syndromes and others. We were particularly interested in TSC and FCDs. And I will tell you very briefly about the genomics of these disorders. It's a simplified version of it.

Dr. Angelique Bordey: 04:54 If you look at the images of the diagram, patients in TSC and some FCDs are born with a germline mutations. It's very few in FCDs. What happened during fetal life is that there is somatic mutations that occur for unknown reason that lead to a subset of cells with increased mTOR activity, and that results in the formation of focal cortical malformations. Here, it's seen in the MRI, the white blebs on the MRI would be in TSC, called cortical tumors or cortical dysplasia. I will refer to them as focal cortical malformation, FCM. These focal critical malformations lead to seizures. Basically, if you remove them through surgical resections, a lot of the patients will be seizure-free. The standard of care as well in TSC also, a blocker of mTOR, Everolimus, that significantly decreased seizures but still has limited efficacy.

Dr. Angelique Bordey: 06:07 Really, the question is, how do we go from these somatic mutations to these malformation in to seizures? This is highlighted here, the goals and question that I will show you during this talk is that you have these mutation and you have epilepsy. When we started this work, there was a big black box in the middle. How do we go from mutation to epilepsy? And I

will show you briefly that we developed a model of focal cortical malformations that allow us to tease apart these mechanisms, and I will present... We ask a question, are these mutant neurons or dysmorphic neurons in these lesions responsible for seizures? And if so, how? And it will present it on a channel called HCN4. Ask a question, a more general question, is HCN4 expression conserved across all of these mTORopathies?

Dr. Angelique Bordey: 07:07 To develop a model, we took the concept that these lesions are fairly focal and they occur during embryonic life or fetal life. So, we use a technique called in utero electroporation, where you can express plasmids in selective neuronal population. This is basically a C-section of the pregnant mom. You get the embryos out and you inject DNA plasmid into the lateral ventricle, then use that current... Sounds gruesome, but use that current and you express the plasmid selectively in progenitor cells that will give it to the baby cells, which are pyramidal neurons. If you do it embryonic day 15, the plasmid is selectively expressed in layer two, three pyramidal neurons of the cortex.

Dr. Angelique Bordey: 07:57 We targeted the medial prefrontal cortex as shown here in its supposed [inaudible 00:08:02] zero mouse, P0, live, where you can really see the fluorescent labeled cells and projection. We chose the medial prefrontal cortex, because it is altered in about 90% of the TSC patients. Then we can do section of these brain and look at the pathology. Again, this is a layer two, three pyramidal neurons, and we can do behavior or video EEG monitoring. To increase mTOR activity, we express a plasmid that encodes a small molecule Rheb that is constitutively active, and this is [inaudible 00:08:40] activator of mTOR.

Dr. Angelique Bordey: 08:45 When we use this approach, we were very excited to find that after doing video EEG monitoring, the mice had convulsive seizures, grade 4 to 5 seizures. Here's an example of the electrical recording and zoom of these traces with interictal tonic, clonic, and oops, caustic dosage events. There's a range of frequency that we can tighter better, and I don't have time to explain how, but as a mean the mice have about four seizures per day. The duration is very standardized at around 40 seconds. So knowing that, we then looked whether our mouse and model display, actually the pathology of the human and to the data I published through several publications. So I go very briefly through it. So here's a coronal section from a mice that was expressing either GFP control or these Rheb to express mTOR activation. And what you can see by eye, briefly, is that in the control the cells, again, line up layer two, three of the cortex and these higher magnification of these cells, pyramidal neurons.

Dr. Angelique Bordey: 10:05 But then the disease condition where our mTOR is hyperactive, the cells are scattered across the cortex. There's also white matter heterotopia, and you can see the cells are enlarged. The soma size is much bigger, which is a readout of mTOR activation, a very well known function of mTOR. And then we characterize all the cells are misplaced, which leads to miss-lamination as shown in the patient. The neurons are cytomegalic, increased cell size, and dysmorphic. Here's an example of neurons that were recorded with patch-clamp electrophysiology, and filled with neuro biotin. This is the control neurons on the left and a Rheb neurons on the right. And you can just see by eye again, and it's quantified at the bottom, we show analysis, that the dendritic tree is much more complex. Then there is gliosis both of astrocytes and microglia. We have shown, and I think others have shown, that there's glial reactivity, secondary to seizures.

Dr. Angelique Bordey: 11:10 And as I mentioned, there is white matter heterotopia. We also know that we do find some kind of cells called giant cells or balloon cells in our section, but they are fairly rare. So we were satisfied with our model that recapitulated most of the pathological features of the human tissue. So on with this model, we're going to ask the question, do mutant neurons can trigger a seizure? We did electrophysiology recording, patch-clamp, and we did find that these which is shown in green in here, these Rheb neurons were actually depolarized. So the resting membrane potential was closer to the threshold to generate action potential, which means they should be more excitable. They are closer to the action potential threshold to generate some. So in light of this data, we decided to normalize the resting potential by using a channel called inwardly rectifying potassium channel 2.1, KIR 2.1, has been used by many others.

Dr. Angelique Bordey: 12:26 So here's a current clamp-recording of these Rheb neurons, and if you inject current, you can induce action-potential firing. In the neurons that express Rheb plus the KIR 2.1, you can see that for the same current injection, they are not firing action-potential. You need to inject much more current to induce action potential, and this is shown on the curve on the right here, the number of action-potential against the injected current. And there cells expressing KIR channels are more sluggish, do not respond to current injection as much as these mutant neurons, Rheb neurons. In addition, which I don't show here, the resting potential was close to normalized. So they are more... We silence these mutant neurons, and to some extent surprising, the effect on seizures was quite dramatic. Basically we almost block seizures. There was a drastic reduction in

seizure frequency. So here is a roster plot. On the top, this is a mouse that express Rheb. The mouse is shown per... I'm sorry... per row, and the columns are days.

Dr. Angelique Bordey: 13:42 So you can see that some mice have seizures every day, some not every day, but all of the mice seized in a fairly high frequency. The mice that express Rheb plus these KR channels to silence the neurons, displayed very few seizures. So we significantly decreased seizure frequency, which tells you that these neurons by themselves are sufficient, or are able to actually trigger seizures. So again, knowing this data, we digged more into the electrophysiology of these cells, usually changes in ion channels or maybe receptors will lead to changing an excitability in cells. So we try to look at what was different in these neurons.

Dr. Angelique Bordey: 14:33 And we were actually surprised originally, the data were a little bit confusing. When we did again a patch-clamp recording in current clamp, the top trace is the Rheb neurons, and here it's a current injection of 300 Pico amp. And these cells fires two action-potentials. The control cells, for the same current injection, fire a lot more action-potentials. So although the Rheb neurons are closer to the threshold for action-potentials, they don't really respond well to depolarization or current injection that depolarize them. And as a result, when you look at that curve, it's again shifted to the right where these Rheb neurons just don't... are very sluggish and do not respond to current injection. And this really tells you that for the same excitatory input on to these cells, they will not generate as many action-potential as your control cells.

Dr. Angelique Bordey: 15:32 So at the end of the day, this is what's telling us that these Rheb neurons are less excitable, which really went against the KIR data. So we analyzed a little more the ion composition of these neurons, and we found a striking difference. Here, our voltage-clamp recording, where you inject current, hyperpolarizing current, into the cells and these control cells responds with an inward current. And this is a fairly small one and the Rheb neurons displayed very large inward current.

Dr. Angelique Bordey: 16:11 And I see a question. Before the current injection test, if it was the question? Yes, correct. So here we do these current injection at the cell resting-potential. I hope I addressed the question. We did... Oops. We did not normalize... Sorry. Well, let's live it that way. So we found this large inward current. This kind of makes sense, these cells are much larger and need to have a lot more ion channels to sustain viability. But what was amazing is that, based on the kinetics of these current, we

speculated that, or hypothesized that there was what is called a H-current or HCN-current. Hyperpolarization cyclical nucleotide gated ion channels, based on the kinetics. And indeed when we block, we used a drug, zatebradine, to block the H-current, there was a significant reduction of this inward current. And this was surprising because these cells, these pyramidal neurons in layer two, three, are not supposed to have H-current. What they have... The remaining current here is an inwardly-rectifying potassium current.

Dr. Angelique Bordey: 17:43 So this was originally surprising to have the abnormal expression of this current. And when we put that drug, zatebradine, which blocked this HCN mediated current, we could see that we actually completely normalized the resting potential of these cells. Completely rescued. So this H-current, HCN channels, were responsible for the cell being depolarized, and this is quite important. What is important as well is that HCN-channels are cyclic-AMP dependent. So they open... They will open or gate by having a hyperpolarization as well as cyclic-AMP increase. So we tested the impact of artificially increasing intracellular cyclical-AMP using forskolin through bath application. And if you look at a control GFP neuron, pyramidal neurons, bath application of forskolin did nothing. Those are an artifact of application. There was no depolarization, but the Rheb neuron, all of them were depolarized with forskolin application and half of them here reached the threshold to generate action-potentials.

Dr. Angelique Bordey: 18:57 So this is a major difference between these mutant Rheb neurons and the control neurons, is that now they are sensitive to cyclic-AMP increases, and that is sufficient to depolarize them and trigger action-potential. So this is quite exciting data that we found. Then to know whether the HCN-channels were responsible for seizure, we needed to know which one. So there are four of them, HCN- one, two, three, and four. So we did immunostaining for all four, HCN3 was not visible. We had HCN one and two, which is usually expressed in pyramidal neurons on the deep layer, not the upper layer, and there was no change, which was really surprising to us. And then we stained for HCN4, and there was a dramatic increase in HCN4 expression, which is actually not there under control condition.

Dr. Angelique Bordey: 20:03 You had some expression in the axons that came from the thalamus, but nothing in cortical, pyramidal neurons. So we were quite excited with this data, and we obtained a lot of human samples to validate the expression of HCN4 in human. I'm showing you an example that is actually published last year. I think it's the wrong citation actually here, but it is published

last year by [Hsieh 00:20:30] and us. And here it's a human sample obtained actually by [Longbo Zhang 00:20:37] and a zoom on the right where you can clearly see that there are some enlarged cells. The nucleus of these cells is enlarged, compare this to the surrounding cells. The nuclei in blue and these cells display a lot of brown, which is the staining for HCN4, compared to the surrounding environment or cells that do not have it. And we quantified in many different samples with also immunostaining and we had similar data, both in TSC and FCD two patients.

Dr. Angelique Bordey: 21:11 So we're very excited with this data. Then we asked another question as well. There are a lot of literature about HCN-channel being expressed after seizures or change by seizures, so we looked whether they preceded seizures. And indeed... We did recording at personal day 8 to 12 and 28 to 42. The control mice don't really have anything and the Rheb mice over at P8 to P12 have increased HCN-current amplitude and this preceded seizures that come around P20.

Dr. Angelique Bordey: 21:44 Then we design a method to block these ion channels using a non-functional, called here NF, non-functional HCN4 channel that acts as a dominant negative that we co-expressed with Rheb. The electrophysiology here shows that indeed we blocked this HCN-current. We rescued the resting potential of these cells. We did not have a dramatic impact on the action-potential firing. And this... The expression of this HCN4 non-functional channel completely prevented seizures, which really indicate to us that HCN4 is important for these cells to be able to generate a hyperexcitability and seizures. So going to the question, is it conserved across all of these FCDs? The answer, we don't know for sure, but what we know is that the expression of HCN4 here, shown by immunostaining in green, in mice. When the mice were treated with rapamycin from [inaudible 00:22:51], personal day one to adulthood, these completely abolished the expression of HCN4. The HCN4 expression is also interactivity dependent. So we would like to think that it might be conserved across other FCDs, this needs to still be validated.

Dr. Angelique Bordey: 23:15 As a conclusion, we gain answers, us and others, on how this mutation may lead to epilepsy. We have now a model to better answer all of mechanistic questions and we found that silencing these mutant neurons was sufficient to reduce seizure activity. We also found that they express an abnormal... They abnormally express an HCN-channel, the HCN4, that seems to drive seizures. These expression is interdependent and I think a couple of weeks ago, Lena Nguyen and showed you that HCN4 expression is actually [inaudible 00:23:56] translation

dependent. Mouthful. So blocking translation was sufficient to reduce HCN4 expression and we hope that this will be conserved across all of these different FCDs, resulting from different gene variants. So the people. A lot other people contributed to this work. Lawrence Hsieh did a lot of the HCN4 work, and he had help from Longbo Zhang and Lena Nguyen and other people started the project like Tiffany Lin and David Feliciano. And we acknowledged funding from NIH, TS Alliance, Department of Defense, Swebilius, as well as in the past as mentioned earlier, CURE, AES, McKnight, and NARSAD. Thank you.

Priya Balasubramanian: 24:48 Thank you so much, Angelique. That was a great talk, very exciting. And before we move on to questions, we do have quite a bit of time so I'd encourage everyone to please send in your questions through the Q&A tab, and we will get to those. So as those questions are coming in, Angelique, I just wanted to revisit the question that came in during your talk and you kind of addressed it, but maybe you want to expand on it a little bit. And the question was whether the resting membrane potential in the Rheb neurons, was it normalized during your current-injection test?

Dr. Angelique Bordey: 25:27 So I assume it was at the... Well, whether it was holding the cells at the resting membrane potential, which we did to inject. We held them at the resting potential and then injected current-injection. Like it would be more physiological condition. I'm not too sure it was a proper answer to the question, but I see it. They are depolarized compared to controls. Sorry.

Priya Balasubramanian: 26:04 Thank you. So we have several more questions here. Do you see HCN4 staining in all FCD-two patient samples or only in a subset of samples?

Dr. Angelique Bordey: 26:19 So far we saw in all of them. Yes, in all of them. We do not know the gene variance for these FCDs. As I know there are 30% or more FCDs that are due to mTOR gene variants, I don't know which one we had. There were classic or FCD-two and I think we reported it in the paper. Some of them had a balloon cell and some did not. So type A and B.

Priya Balasubramanian: 26:58 Thank you. And here's another question. Do you see a translational path, and I had the same question, do you see a translational path to treat people?

Dr. Angelique Bordey: 27:09 So I think, yes, the answer is yes. So the beauty of the HCN4 is that it's not in the cortex, at least in adults or young adults. So it is a perfect target for gene therapy. It is expressed during

development, it has not been studied very much, but it is there, and after birth it decreases tremendously. It's expressed only in the thalamus, I think the amygdala and the cerebellum. So if targeting the cortex is a good gene therapy target, then can we use a drug? Presumably not because that would slow down the heart, the HCN4 is expressed in the heart and is important for their pacemaking activity, which I did not say, there are pacemaker channels, which also explain why they would maintain seizures, activity or cell firing.

Dr. Angelique Bordey: 28:08 Can we consider antisense oligonucleotide? Possibly. I think there is a lot of development by companies with small SIRNA that can be delivered intrathecally and they will last for about six months. HCN4 may be a good target. I think we need to know what is the function of HCN4 in the thalamus, because we would not want to block it there. It may be in the hippocampus a little bit. So it would be worth trying. I think it's important actually to try whether a systemic SIRNA injection for covering the brain would work and have no side effects. So this is a big question. It's just easier than gene therapy to move towards clinical application. So those are the two alternatives I can think of.

Priya Balasubramanian: 29:01 Have you tried blocking HCN4 after seizure onset?

Dr. Angelique Bordey: 29:08 We did not. This is a very good question. We wanted to do it. We have the plasmid, we have not done it. We know you can block... When seizures are established, we know that we can block them and it is sufficient to... We can inject drug and it's sufficient to block seizures. We have done that with Lena who showed that blocking translation after the onset of seizures, after they establish well decreased seizure frequency. So you can shrink the cell size and presumably remove ion channels since HCN4 is translation dependent. We have also shown that if you block a molecule called [philomena 00:29:54], and we published that last year. If you block the activity of philomena with a small molecule, once the seizures are established, so in mice that are, I think there were maybe two months of age, you do decrease seizure frequency. So I'm hopeful that doing that also in adult will work.

Priya Balasubramanian: 30:17 Here's a more general question. Do these genetic epilepsies respond well to vagal nerve stimulation or resective surgeries?

Dr. Angelique Bordey: 30:26 So I think they're. Yes, resective surgeries. Yes. They respond well. I think they're... I don't know all the numbers on top of my head, but maybe 20 or 25% of the patients will go through surgeries, I may be wrong with the number, but roughly. Not

everybody can go through surgeries, depending on the location and if they have too many of these malformations. Patients can have 1 to 50, which makes things complicated. And I think at least 50% of the patients will be seizure-free, I think. I think it depends on the clinical center, maybe 50 to 70, but very often seizures come back. So that's a problem with the surgeries. The vagal nerve stimulation, I know it is used, and I do not remember the numbers, the test statistics in terms of efficacy. So I don't know that.

Priya Balasubramanian: 31:29 Do you know the upstream genetic drivers of increased HCN4?

Dr. Angelique Bordey: 31:38 So like I said, we know it's rapamycin sensitive, we saw it. So we use the plasmid Rheb, and Rheb is right upstream mTOR, and this is sufficient to increase HCN4. We have, I don't think we published it, but we had a [inaudible 00:31:57] in TSC one [flocks 00:31:59] mice, so mute knockout mice. In TSC one, HCN4 four was increased as well. And since it's increased in the patient, presumably it includes other genes since TSC one and Rheb, and then upstream, you have AKT and PI3K. But we have not looked at PI3K, we have not looked at P10 [inaudible 00:32:23] five, for example, to get to one complex, which I think is really important to do.

Priya Balasubramanian: 32:28 Do you know if it's translational or is it the transcription? The up regulation?

Dr. Angelique Bordey: 32:36 So it's for ADP dependent, like I think Lena had shown, so it's translation dependent. There is no increase of the MRNA, at least what we saw in our mice. And it's not unexpected that you have cells with floating MRNA that is not translated, so we clearly promote a translation of that gene.

Priya Balasubramanian: 33:01 Oh, here's another question. Is the HCN4 MRNA the same isoform as found in other parts of the brain or the heart? And if not, there may be potential for targeting [inaudible 00:33:14].

Dr. Angelique Bordey: 33:14 Yes, this is a good question. And I've asked myself that question and we have not, we have not checked. It is a possibility. So there are two different isoforms, a long and a short isoform, and I don't remember which one the brain has, but the problem is we have not looked in the disease condition, which could even be a slightly different isoform. So I think that this is a very... This is something really important to do, I think.

Priya Balasubramanian: 33:47 Okay. That's the questions we have. Just one final question was, do you know whether HCN4 is associated with other types of epilepsies? And maybe you touched on this already, but just to-

Dr. Angelique Bordey: 34:06 Yeah, actually I don't remember the group that did it, but they found... I actually don't remember right now, I should have checked. I know there is a mutation in HCN4 channel that has been reported, I think maybe last year, that is associated with seizures. I don't remember if it's a gain of function or loss of function. So there's one study that reported that. There's also genomic association studies showing that it actually could be associated with also a bipolar disorder since you control excitability. So I think this is quite... This is a gene of interest that people have not seen in the past because it's a very discrete expression in the brain, so people thought very limited function and you have no clean blocker of HCN4. The blockers will touch all the HCN channels, so it has been hard to study by many. But now I think that our study highlights the importance of this channel in seizure, and perhaps, other disorders.

Priya Balasubramanian: 35:16 So there are no more questions. So I will take this opportunity to say thank you again to you, Angelique. And I also want to say thank you to the Nussenbaum-Vogelstein family for their generous support of this Frontiers in Epilepsy Research Seminar Series, and thank you to the audience for joining us and for asking questions. If you would like to have more information about hosting a seminar series at your institution, or applying to one of our grants, you can visit our "for researchers" page on our website, or you can contact us at research@cureepilepsy.org. Keep an eye out for our Fall 2021 seminar series announcement. We will be releasing that over the summer. And finally, we really value your feedback. So please do take a minute to take the brief survey that will follow after the seminar. Thank you.