

MERRITT-PUTNAM SYMPOSIUM

Let us get started. I would like to begin by first welcoming this year's presenter for the Merritt-Putnam Clinical Fellowship Award for 2005, Dr. Gregory Barkley. Dr. Barkley is on staff at Henry Ford Hospital in Detroit, Michigan. He also serves as Chair of the Epilepsy Foundation's Professional Advisory Board and the Epilepsy Board of Directors. Dr. Barkley is an Associate Professor of Neurology at Wayne State University in Detroit, Michigan. Please welcome Dr. Barkley. (Applause)

(Dr. Gregory Barkley)

Thank you, Dan. On behalf of the Epilepsy Foundation, I want to thank Pfizer for its long-term commitment to the Merritt-Putnam Fellowship Program. This work has been vitally important and has been going on for more than 20 years. Pfizer also supports the mission of the Epilepsy Foundation in a number of different ways, and we are grateful for that support as well. Among the things that they support are the Speak Up Speak Out Program, which is a grassroots advocacy program and a public policy institute where every year people with epilepsy and their families come to Washington to speak out on behalf of legislative issues to try and bring more support for research in the field of epilepsy. Pfizer also supports other programs that have to do with increasing the understanding and fighting the stigma of epilepsy. It is an honor for me to be here today to present the Epilepsy Foundation 2004 Merritt-Putnam Clinical Research Fellowship. This fellowship is awarded based upon a scoring system that determines the applications and merit. The application score is determined by a review panel comprised of experts in the field of epilepsy. The Merritt-Putnam Fellowship funds one of the highest ranked research programs in all the applications that we have received. The first pilot fellowships of this program were funded through Pfizer in 1981, and the initial recipients were Blaise Bourgeois and Robert Leroy. Since then, a distinguished group of epilepsy researchers have been awarded annually, and we have another one who we hope is going to start a career that will join this distinguished list.

This year, I want to introduce you to Eric Marsh. Eric Marsh is going to study in his fellowship malformations of cortical development, and he is going to be doing this at Children's Hospital of Philadelphia. Eric, would you please come up here. (Applause) Congratulations.

(Dan)

Congratulations Eric, thank you, Greg. I would now like to welcome this year's presenter for the 2005 Victor Horsley Fellowship, Ashley Gaines. Ashley is a Senior Marketing Manager in Pfizer's Neurology Group. She is responsible for a number of the initiatives that underscore Pfizer's commitment to epilepsy. In this capacity, she works closely with the Epilepsy Foundation to make fellowships like Victor Horsley possible. Ashley. (Applause)

(Ashley Gaines)

Thank you. Good morning, I am Ashley Gaines, and I work in the Marketing Group of Pfizer. It is my great pleasure to be here today to present the 2004 Victor Horsley Fellowship Award to Dr. Nathalie Jette of Columbia University. Dr. Jette is precepted by Dr. Martha Morrell and Dr. Helen Sharpman.

Pfizer is committed to the support of cutting edge research such as Dr. Jette's work focused on the relationship between brain-derived neurotrophic factor and estrogen in women with epilepsy. As many of you probably know, certain types of epilepsy such as juvenile myoclonic epilepsy developed at puberty and up to half of women suffer from catamenial epilepsy, highlighting the importance of sex steroid hormones in epilepsy. Moreover in most animal studies, estrogen has found to be epileptogenic. And recently a role for brain-derived neurotrophic factor BD&F and epileptogenesis has been proposed. As well, as a putative estrogen response element in the gene encoding BD&F. The aim of Dr. Jette's research is to test the hypothesis that estrogen increases BD&F expression, thereby leading to an increased risk of seizures in women with epilepsy.

It is truly an honor to present the Victor Horsley Fellowship Award for such important and exciting research. On behalf of Pfizer, I want to thank you for your contributions to advancing the field of epilepsy. Congratulations. (Applause)

(Dan)

And now it is my pleasure to officially start the 24th Annual Merritt-Putnam Symposium. As many of you know, this is one of the great traditions of the American Epilepsy Society, a symposium that is intended to try to bridge from the bench to the bedside, bring science to the clinicians and vice versa. And I am very pleased to welcome Dr. Jeff Noebels who is Professor of Neurology, Neuroscience and Molecular Genetics at Baylor, who is now the Chairman of the Merritt-Putnam for this year and the next 2 years. Jeff, welcome. And we are looking forward to a great symposium.

(Dr. Jeff Noebels)

Thank you, Dan, and it is a pleasure to follow in your footsteps. This really has been a wonderful symposium track record. So thank you all this morning for coming to the 24th Annual Merritt-Putnam Symposium this year on *The Developing Epileptic Brain*, and thank you also to the American Epilepsy Society and for Pfizer for their educational grant that makes this possible.

Now I have to read you some notes for CME purposes. It is a mystery to me why I have to tell you them again and, unlike most mysteries, this one gets more boring as it goes along. It is the policy of the American Epilepsy Society that all faculty participating in continuing medical educational activities are expected to disclose to the audience any conflicts of interest. Faculty disclosure has been made in writing in the syllabus. Each speaker has been asked to disclose to you if and when unapproved products and/or unapproved indications will occur in their presentation. AES now has the medical education evaluator, an online system to obtain CME credit. This is new, and it is important. The evaluator will be used to verify course attendance and your pre- and post-

tests and complete course and activity evaluations. You can use the preassigned numbers printed on your badge to gain access to the activity and course evaluations. A booklet containing paper copies of the test and evaluation forms are available in the conference bag and also will allow for note taking during the test. To receive pharmacy credit for this activity, please complete the pharmacy credit form included in the program syllabus. Please return the pharmacy credit form to the registration desk outside the ballroom before you leave, and the certificates will be mailed to you. Now at the end of each talk, question cards which are present in your symposium booklet can be passed forward and at the end of the morning's lectures, we will be able to deal with the questions in a panel symposium period. Very good.

Well, this morning we focus on epilepsy and the developing brain. This is something that is near and dear to me and also one that is complex. The complexity arises from the interrelationship between the two; both are proceeding at their own separate tempos to change the brain and at different levels at different paces. A lot of the molecular events are interfering with each other. And so the challenge becomes our need to delineate what is causing what. Are the seizures changing the brain? Yes, they are. Is the developing brain changing the pattern of seizure activity? Yes, it might be. Now this turns out to be an extremely difficult symphony to dissect, and fortunately, we have an ever-growing toolbox of new technologies to dissect them. Although you might think that this is an impossible task, I have learned one thing as a scientist and clinician is never say never. Never, never say never. And I think what you will see is from the five superb speakers that we have this morning, that we are making real progress in understanding some of these early events in the developing brain and how we might begin to ask the kinds of technical questions that at some point will allow us to manipulate and interfere, hopefully prevent and protect brain cells from the kinds of changes that lead to an epileptic phenotype.

We have five speakers who are going to be taking us through different levels of organization in the nervous system and the best place to begin is with the kinds of images that we are capable of seeing now of the developing brain. And then we move on to consideration of building the epileptic circuitry. So how do we construct cortical circuits in the brain? How do we then go on to add the essential inhibitory components, the interneurons, to this network? And most important, how is the membrane and molecular excitability and signaling patterns in neurons changing over time and in response to seizures themselves? Then we will move in two final talks to some real life biology of epilepsy, which are some of the problems of trying to understand the natural history of some early brain changes, and then, finally, some of the molecular pathways and indeed targets for potentially protecting injury in the newborn brain.

So I think we should get started with our first speaker who will start us off. It is Ellen Grant. She is Director of Pediatric Neuroradiology Imaging at Mass General Hospital, and she will talk about what we can see now. And I think as you see her talk, you will realize that you do not need to be a fortuneteller to predict the future. We will see what we are going to be seeing soon using the latest technology. Ellen, welcome.

(Dr. Ellen Grant)

Thank you very much, and thanks for the invitation to speak here. So what I am going to talk about today is some of the imaging techniques we are using to look at the brain, both on a macroscopic level and to start to probe the microscopic structure of the brain.

So the four areas I am going to focus on is first, how resolution imaging began, the macroscopic structure; how we are going to look, use that data to look at brain growth which has implications on circuitry development; moving on to new techniques to look at white matter organization, and cortical organization.

We are starting with the high-resolution imaging. We have shown before that phased array imaging, which includes a signal to noise, helps us in detecting lesions in patients that have epilepsy. This is more true, I think actually, in the pediatric population, where there is often malformations in cortical development. And then the studies we have done previously, and this is now old data and has been reproduced recently in similar data from the group at Cleveland Clinic that we see improvement in detection of lesions, and the diagnostic information added is significant.

Here is an example of the type of structures that we are able to see, and here is a small transmantal dysplasia. The left frontal lobe of a small girl about 6 years old seen here in the middle image has an area of increased T1 signal, but you could not see it on the standard head coil data. So the increased spatial resolution is allowing us to detect the structural alterations in children with epilepsy. As the resolution in this particular lesion is about 5 ml in diameter; so we are getting to this subcentimeter level of detection in detecting focal anomalies. Now, we have been duplicating that data at 3 Tesla with the hope that with advances in phased array technology, we will be able to probe even further into this spatial resolution.

In a recent study that we did looking at the 3T data, we found about a 45% improvement with relevant clinical information by looking at this data in terms of improved signal to noise. So we do get similar improvements. It is difficult to compare the two studies because the population that we looked at in this next study at 3T was referrals from the whole Boston area from tertiary care centers; so the tendency was to refer more complex patients.

Here is an example of the type of lesions we could see in the 3T data. And in the left motor cortex, just look at the hand representation, you can see a little area of increased T1 signal and blurred gray-white junction. This lesion again in size is about 5 cm in size and very ill defined. You could not see it on the standard images but, the resolution and the sharpness of these images allows us to detect these small abnormalities. So in general, the best results we get is by working as a team getting the best resolution images we can with the phased array technology, 3T imaging, targeted study, and collaborating together so we have the best idea of where to search these datasets.

If you look at the signal-to-noise estimations, you can see that as we move up to 3T with surface flows, we are getting up to about an 8 times signal-to-noise improvement compared to the standard head coil. Now we have not fully realized that 8 times improvement in signal-to-noise, but this is where I think we are going in the future. Currently, the phased array technology at 1.5T is pretty good, and we are actually running 23 channel coils. And some are very experimental coils, have 90-somewhat channels in them. So the signal to noise is significantly improved and even at 1.5T. The phased array technology at higher tesla is 3T, and 7T is lagging. But as we get better with the phased array technology, we will be improving our signal to noise; it will allow us to probe a better spatial resolution. Currently, our spatial resolution for these anatomical datasets are about 400 μm in plane, and sometimes we can push it down to 200 μm .

Seven T imaging, again we are very excited about, is a potential way to see micron level structure. So we are using 7T technology to try to get at the micron resolution, at least 100 μm resolution for the structural datasets. And this is an example with similar head coils with 3T compared to the 7T dataset. And you see that on the 7T dataset, the image is much crisper because of the significant improvement in signal to noise.

Well, we are starting to look at animal studies at the 7T level. If you look in the poster aspect of this image, you can see a band between the white matter and the outer edge of the cortex, the **salina genari** [??]. So we are getting spatial resolution at 7T with just a standard head coil in the 200 μm range in live specimens. When we start to look at post specimens at T, we are getting down to the 120 μm isotropic. Those last images I showed you were 3 mL or so in thickness. Now we can with post specimens at 7T get down to 120 μm isotropic, so in all planes. So each box in this image is 120 μm and you can see this spectacular detail of the hippocampus and the CA1 sector, the subiculum, in the detail that we have not seen previously in anatomical MR imaging. Our hope is that once we get to the multi-phased array coils at 7T, we can pull the imaging times down and start to do this type of resolution on real live patients. And once we have got good datasets, and we can see structural anatomy well on the MR images, we still are at the 100 μm level and that still is far too large to look at the circuitry and the organization of the cortex or the white matter. So we need to do these other things to also get an idea from the imaging data how the brain is developing and the differences between normal and the epileptic brain.

What we are doing currently with normal newborns to look at this developmental stage, and this will be again moved over to ones with disease processes such as epilepsy, is to segment the brain and look at the different structures. In this set of images, show a coronal 3D T1 image in the top plane that has been segmented on the right by a manual segmenter and then color coded as to the different regions that we partition these brains into. Now using that data and, it is a bit of a busy slide, we can look at growth, and this is just between the gestational weeks of 30 and 44 weeks in children born premature through postphase. We can see linear growth in some of these regions and when we compare this blue line on the top is pathological specimens that were fixed and then studied to look at the volume of the hippocampus. Our data, because we don't use specimens, has smaller volumes in general, but track very well with the gross curves of

the hippocampal data from the Ompresh-Ven data sets. This is really exciting to us because now we can look at the growth of these small nuclei in newborns through these early stages and follow it into the infant and young childhood age and look at the growth of the different nuclei in the brain as well as the entire brain overall. We can also subsection the brain into different lobes and look at the differential growth of the different lobes and you can see on this the frontal lobes very much grow more rapidly than the limbic lobes.

Looking at surface representations, we can also look at the development of the complexity of the brain and its gyral-folding pattern, and these are just surface representation data through the small time period of 31 weeks through just after 42 weeks gestation. And it is easy to appreciate the growth and the increasing complexity of the brain. But the problem is when you have that complexity, how are you going to analyze it. So what we have done with our datasets is take these surface representations, or take this segmented brain that is actually a bunch of lines in a 2D image, and put it into a 3D analyzing program called FreeSurfer™, where the brain surface is represented as an entire surface. This image here, what we have done to get this image is to inflate the brain, and we tag where the sulci and the gyri are by color coding so the depth of the sulci are coded in red and the apices of the gyri are color coded in green. Now that we have the whole surface representation, we can start to do measures that we have not done previously such as look at the surface area, the gray-white junction, which is this column here. And you can see how it increases through the gestational age. And I have put an adult controller here at the bottom to give you an idea of the range of growth we are going to see in these kids. We can also do very accurate measures of cortical thickness, where at each point in the cortex, we look at the shortest pathway to the pial surface from the gray-white junction and calculate very accurately the cortical surface throughout the brain. That is this middle row here.

Also, we can develop measures of cortical curvature. This last column is actually what is called the integrated doubling perimeter, which is a measure of how much the cortex is folding, and we are working on a number of different parameters to better encapture the complexity of the gyral folding pattern. And you see how this increases again with gestational age and is much larger in the adult. We can also probe these datasets regionally; so you can look at just a frontal lobe, a subsection of the frontal lobe and get parameters of these same types of, with the same data, in subsections of the brain. So we are very excited about this and one of the things that I thought was very interesting: if you look at the standard deviation in the cortical thickness in the younger brains, it is actually much smaller than the standard deviation we see in the adult range. And to me that is very exciting as well, too, suggesting that cortical thickness is much more uniform in the immature brains and as the children grow they start to get cortical subspecialization that leads to differences in cortical thickness and hence the larger standard deviations when you get to the adult time period.

The reason this is so important, and I will show an example from a paper on ADHD. And what we are seeing here, is people have been tracking volumes of subcortical structures in the older patients. Here they are starting around 6 or 7 years of age, and it is

helping us to understand how different brains are organized differently. So in other words, if you took this dataset and looked at kids with ADHD when they are 18 or so, you would not find any difference in volume; for example, the caudate nucleus. But if you track the growth of that nucleus from the 6-year age group up, you tended to have very different growth curves. And this is at the other end of the growth curve and what we are looking at, but it does make the point that it is not only important what volume a structure ends up at, it is actually the growth pattern it follows to get to that point. And there is a lot of implications in terms of how the nuclei are hooking up to create neuronal circuits that is embedded in this data of growth over time. So longitudinal growth of different nuclear substructures and how they coordinate has strong implications into how neuronal circuits are being developed. And this is something we are probing now in the newborns through the first few years of life to try to understand what the normal parameters are for growth of these subnuclear structures and what happens in diseases that are either genetic or epilepsy disorders that occur due to injury at birth.

This is a little summary slide to point out what I just mentioned that there are strong implications for developmental organization in the neurocircuit with this growth data. It is important that growth curves are as important, if not more important than the final volumes, but we need to also develop the ability to quantify complex shapes such as a cortical surface, and that is something we are actively working on now with groups of theoreticians from MIT.

Can we use these techniques to define genetic phenotypes and to understand global effects of epileptogenesis. And I think that's the big challenge to the group here is, we are developing these techniques, we are starting to develop normal trajectories; what we need to do now is to start adding in those abnormal, the children that we know have a high probability of developing epilepsy to see how different the growth curves are and to see if we can understand better what circuits are involved in development of epileptogenesis. And this is now expanding to involve not only the focal epilepsy obviously, but also the generalized epilepsies.

So now we can look at growth again, and we can look at anatomical structure down maybe if we push really hard with past specimens to 120 μm or so, but still that does not tell us anything about how the brain is wired up. It does not tell us anything about cortical organization at the cellular level and that is something that is very interesting to understand and has big implications in terms of understanding and interacting with the microbiologist.

One type of technique that we are using to probe white matter structures is diffusion tensor imaging. And what I have shown here is an image from a diffusion tensor dataset, but it is a fractional anisotropy image. On this image, areas that are very bright such as the corpus callosum and you see the genu of the corpus callosum anteriorly that I have labeled as bright. The brightest area is white, is a value of 1, meaning that almost all diffusion is in one direction. In areas that are darker, and the darkest would be something like CSF, where diffusion is the same in all directions, there is no directional bias so the FA value is 0. So in this dataset here, the data range is from 0 to 1:0 being completely

isotropic, to 1 being primarily diffusion in only one direction. So using this type of data, we can start to probe the organization of the white matter and to look at the density of white matter connections and the coherence of white matter connections.

There is maturational changes in these datasets. And I have shown here a group of patients that range from birth through 8 months of age, and you can see that the brightness of the FA match changes over time, particularly in the subcortical regions as some myelination starts to develop. And interestingly enough, the FA changes tend to occur before we see T1-weighted changes, which are changes due to the development of large macromolecules around the myelin. So there is something about the initial physiological changes that occur that result in FA changes very early on.

Then we've been probing the tuberous sclerosis patients and trying to understand how does this white matter organization help us understand seizure disorders. And one good example is the tuberous sclerosis complex. So we know that a lot of these patients have cortical tubers, but often there are white matter abnormalities associated with it as you can see in this patient. You can see tracks of increased T1, T2 signal that track down to the ventricular margin. And we suspect that in a lot of these patients, even areas of white matter that look normal probably have slightly different organization, and there may be at least two different categorizations that correspond to the two different tuberous sclerosis genotypes. Now if we take these patients, and what we have done recently with Elizabeth Keogh is, you take her patients and look at ones that are cognitively on track and compare them to patients that are cognitively off track. And what we find when we segment out the deep ray structures. So the image we analyzed was the image on your right on the bottom panel, this image here, and what we have done is took this image and created a histogram of the FA values of those entire brains taken off the cerebellum as well. So looking at the cerebrum and looking at a histogram of the FA values, again 0 meaning little coordination, or few tight compact bundles that are unidirectional, 1 meaning that there is a lot of areas that have very tight coordinated white matter tracks. And what we find, which is statistically significant in these patients, is we caught cases that are cognitively off-track, start to draw up their high FA values. So the green curve are the patients that are cognitively on track; the blue curve is the patients that are cognitively off-track. And you see in the higher FA values that the green curve stands above the blue curve. So we are dropping the number of tightly packed coordinated white matter bundles in these patients that have tuberous sclerosis that turn out to be cognitively off-track. Also statistically significant in this small data set was the mean and the kurtosis, so we are seeing this FA data is giving us some information into brain organization and how it coordinates with cognitive function.

Interestingly enough, we did do a statistical test to correlate with one of their TSC1 or TSC2 genotype, and that did not have any statistical significance. Then once we have this data, and we say, well the diffusion is primarily in one direction versus uniform in all directions, we can take the data and also look at what direction the diffusion is going in. And what I am showing here is a color-coded image of the diffusion direction in the white matter superimposed on an anatomical scan. Green codes for anterior to posterior; blue, superior to inferior; and red, actually did not come out on the slide here, is left to

right. So you can see in the corpus callosum in the image in the coronal section is going left to right; the cortical spinal tracts are blue; and some of the anterior posterior fibers in the frontal lobe are coding as green. So we can also not only look at the coherence of the white matter tracts, we can also look at the directions of the white matter tracts.

If we take each voxel in that image that I just showed you and represent each voxel as a box, color code it as to the primary direction of the diffusion, and the box is the size of the magnitude of the diffusion, and the more rectangular the box gets, the more the diffusion is in one direction. So as a box gets more and more rectangular, the FA values are actually going higher and higher towards 1. And in this image here, you can see I have blown up a portion of the brain in the right posterior quadrant in the parietal zone, and you can see these boxes pointing in different directions. But we can, if we see a bunch of boxes connected together and going all in the same direction, we can develop rules to say this is where a tract should be. So if I see two boxes going in about the same direction with, for example, less than a 30-degree deviation in their major direction, I am going to assume that is a tract and those two boxes are connected.

When we do that we can start to do what is called tractology, and I am going to show you in this image here; where this falls down is in areas where there are crossing fibers, and if you look in the area just posterior to the green region and just posterior to the blue region, we have crossing fibers. So in the east box, we are averaging axons or water motion in more than one direction together. So if you are averaging two different directions that are 90 degrees to each other, for example, the average is going to be essentially 0. So actually 45 degrees, but a much smaller quantity, and if you get more directions averaged in, the more and more you lose the directional information in that dataset because of the combination of multiple directions.

So now if I take this image and I hook up these boxes to create a tract, I can create images that are called tractology, and here are a couple of examples of images. The left two images are in newborns, where I can pick up the cortical spinal tracts quite easily; and the ones on the right are adults, where I pick up a former complex arborization of the cortical spinal tracts. So these are very interesting. We can look at tracts to a certain degree here, but all the interesting data by crossing fibers is lost. So we actually do not use diffusion tractology that much in the epilepsy population; we have actually moved on to a different technique, which I am going to explain to you now.

Now I have told you about when we have the two different directions in the same block; so for example, if you imagine two different fibers here, if I average those together I am going to get a very small diffusion in a direction that is not the actual correct direction for the diffusion fibers. So what we do now is, we do something that is called diffusion spectrum imaging that was pioneered by Van Wesselen, and what we do is a probabilistic map of where diffusion is likely to occur. So instead of saying in one box that we image, there is only one primary direction to diffusion, what we do is we look in 360 degrees and calculate the probability of diffusion at each angular direction, and that allows us to develop probability maps of where diffusion is likely to occur. So when we do that, we see a big lobe of probability in this direction. In the right corner, I am pointing to the red

direction, and in the same collection you can see the middle lobe that has green. So we have two different fibers – one going left to right and one going AP – that we are resolving by doing this type of technology. So it is just basically a probabilistic map is the simplest way to think about it in 360 degrees looking for lobes of high probability, which we assume are the areas where fibers are going.

By taking that dataset, these are the type of images we are creating. And you can see I have put up here Jeff Noebels' last year for my last talk last year, sent me Wendel Creed's manuscript, and you can see how the white matter tracts that was described back in 1963 by Laboyer's dissection probably taking months to years of time, we can do now in about a half an hour with current diffusion spectrum imaging technology in 2004. What we are seeing right here is those crossing fibers. You can see the cortical spinal tract coming down the corpus callosum fibers intersecting, and we are starting to be able to now resolve the complexity of the crossing fibers, which is really probably not the true anatomical structure of white matter complexity.

This is a video clip of a dataset obtained on a normal volunteer. We imaged the superior half to the brain by the level of the bodies of the ventricle superiorly. Red again is left/right so you can see corpus callosum fibers there. Green is cortical spinal tract primarily, and green are some of the association fibers running anterior to posterior. So as this rotates towards you, you can see the profound complexity of these datasets, and actually our biggest challenge right now is to figure out how to analyze them. We can look at them, but coming up with a way to quantitatively analyze them is the biggest challenge. The interesting part about this is although our voxel resolution is about 2.3 mm³, what we are probing actually is a space in the order of microns so the diffusion times we use is, again, allows water molecules to move only a few microns in distance. So although our spatial resolution is 2.3 mm, we're probing structure of the white matter, an organization of the white matter, probably at the micron level. And we have been playing with this technology and some of the malformations to see what we can see in areas that are malformed to help us come up with again quantitative measures that pull out the significant differences in organization that we see in patients with epilepsy. And I think that this is going to be a very important part; I think we are all starting to understand better that the white matter probably plays a significant role in epileptogenesis and development of abnormal circuits. So understanding how disorganized the white matter is or where the connectivity has become abnormal outside the cortex is also very important.

And in this particular dataset we see a patient with a focal cortical malformation on the left, which is an area of increased T2. The DSI dataset is shown on the right, again color coded green to superior; left anterior to posterior is green; superior to inferior is blue; left right is red. The interesting part to me, too, you can see a little dark band on the T2 image running anterior to posterior and the image on the T2 image which corresponds to this association fiber in green on the DSI dataset on the left. And what this is starting to show, and I think what we are starting to probe, is a differential involvement of different types of white matter tracts. And I think that we are going to be able to start to pull that type of information out. Is it the association fibers that are primarily disrupted in some of

these malformations or is it other more distant fiber tracts? Is it more the structure, short-water structure that is disrupted in terms of centimeter organization or is it again the long tracts that are involved? So this is allowing us to probe that type of involvement of white matter and selective white matter involvement, and I thought it was interesting we did see preservation again of the association fibers. In this region here we probed it fairly extensively in a 3-D dataset. You actually have to pull these up on a screen and rotate them in 3-D to really start to get at the complexity of it. We see a drop-off in a lot of the connections between the contralateral hemisphere when we compare it to normal. So again, we are starting to probe differences in the organization of the white matter.

This is a collection of patients with tuberous sclerosis complex. I am showing a level of flare images just above the ventricles on the bottom panes. All these three patients are from the same family, so presumably the same genetic defect. All have different phenotypes, however, and you can see just on the coloring of the DSI datasets above them that each of them looks slightly different; so we are also using this DSI data to probe the genetic phenotypes of different patient families and hoping this is going to help us understand the phenotypic variation and the role these genes play in the patients that do develop epilepsy.

So if we go look at white matter organization, can we also look at cortical organization? And some of the first people to start to think about that were McKinstry and Jeff Neill's group at Wash U. And they had a paper in *Cortex* showing diffusion orientation along radially in the cortex, which you can see in that box blown up in the bottom right-hand corner. If you look at where the cortex is, the organization diffusion goes pretty much in the radial direction in those areas and it's thought that that radial organization is something that we can probably pick up. When we look at our datasets, and unfortunately I could not bring all the data images here today, because it is such new data that my collaborators did not want me to show it publicly yet; you can see even in these datasets, this surface is showing the gray-white junction. And you can see sticking through the gray-white junction these bands that are radial, and we find this radial organization throughout the cortex on our diffusion images, and it is a very strong signal that we see even through to the adults, this very strong radial organization, which is a scaffolding of which the cortex is developed on. What we have also tried to do is probe the horizontal organization, the laminar organization, of the cortex.

And this next image shows how the horizontal organization of the diffusion changes and different regions. So in the region here that is circled in green, you can see that the discs are all oriented along the gyrus, which is actually the motor gyrus, I think sensory gyrus – sorry. As it will organize along the gyrus, we are seeing organization of the horizontal architecture of the brain cortex that seems to map to cortical functional areas. So I am very excited about this, and we are now optimizing these techniques to probe the cortical structures of areas such as the cortical tubers in our TSC patients, trying to understand what is normal cortical organization and how is it altered in patients that have malformations. And ideally, it would be nice to look at the younger patients. Currently, however, these scans run on a 3T machine, and I cannot run sedated patients on that particular machine so I am a little limited as to how young we can do in terms of doing

these types of scans. The time these datasets take, we can do it in about 15 to 20 minutes. We get better datasets in 30 minutes; we can do great data, fairly excellent datasets in about 45 minutes. So it does take a bit of time.

In the animal studies, what Van has done is taken this horizontal organization, and see how it mapped to cortical parselation areas that have been identified in animal atlases, and here is a comparison. I think it is a mouse comparison of cortical organization color coded by the horizontal direction of the diffusion on the left, and on the right you see the organization of the cortex as defined by pathological studies on the right. So we can do animals actually, and this is a study done by Van Wedeen. Also working with him on this one were Alex Discrespny and Flore Nycler, looking at rabbits. And if you fix the datasets, we can actually get 100 μm resolution for the standard images. The one I am showing here is T2 weighted imaging on the top, FA map, and then an ADC map. And we can create DSI datasets from these animals and look at the organization of the white matter and the cortex in the animal specimens. The important part about this is this is actually a profuse fixed animal; so we can run these in the scanner overnight and start to probe the structure of the cortex and the white matter organization in knockout animals. So again this another, I am very excited in terms of a way that I can interface with some of the following speakers that are looking at the microscopic organization of the brain, that we can probe similar mice and knockout mice to look at how that alters the white matter organization, the cortical organization. And this is the type of organization that we have not been able to get at previously with either path datasets or with imaging prior to this. So this is again I think a very good, significant advance and again pioneered primarily by Van Wedeen and our center.

So we have moved now from just seeing these cool little small things in the cortex on anatomical images in the order of 5 mm or so to these type of images, the DSI images on the left, which I think are truly probing structure at the micron level, and are going to I think help us really expand our knowledge of cortical organization in vivo, which I think is going to be a huge advance. Not only are we looking at the knockout mice, but also trying to translate these datasets into humans, because the human data is never quite like the animal model. So we are very excited about the potential that this is going to provide us.

In closing, I would like to acknowledge the NIH support that we have; again, research funding is extremely crucial to moving things forward. And in particular, Van Wedeen's funding to work on the development of this DSI technology. And I would also like to acknowledge that the large group of collaborators that I do work with that I have seen working at a large center like Mass General Hospital, you have an almost infinite number of physicists and clinicians and physiologists who work with, and it opens up a lot of doors for really pushing the edge in terms of looking at the interface between imaging and the biological subspecialties.

Thank you. (Applause)

(Dr. Jeff Noebels)

Thank you for that spectacular talk; it makes me wonder whether we should start referring our patients to physicists from now on, at least first. About 100 years ago when you would try to take a picture of something, you had to ask the person to stand perfectly still for a long time. Actually now, it is quite the opposite and we have these beautiful digital video DVDs of things that we try to understand.

Our next speaker is Professor Arnie Kriegstein who is currently Professor of Neurology and Director of a Stem Cell Center at the University of California and San Francisco. He has been spending a lot of time analyzing the earliest events of cortical formation and has some really spectacular movies to show us of exactly how this occurs. Arnie.

(Dr. Arnie Kriegstein)

Well thank you, Jeff, I certainly appreciate your invitation to talk today and my topic for the next 20 to 30 minutes is going to have to do with very early stages of cortical development, and I will focus mostly on the development of the excitatory pyramidal neurons in the cortex and touch upon how they hook up with the inhibitory interneurons to form some of the canonical circuits characteristic of cortex.

So we will begin by talking about where the cells come from – the neurogenesis, especially the excitatory cells. And the bottom line is that radial glial cells, which had long been thought to be static cells that provide a scaffolding for brain construction, are actually neural stem cells themselves that generate the elements of the developing cortex. And the rodent model that we are going to be using, unlike the human images that you just saw before, are shown here in coronal section with the front part of the forebrain cut off. To emphasize the neurogenesis, the period of time when all the cells in the cortex, the neural cells are generated is an embryonic event that ranges from E12 to E18, roughly the overlap between the first and second trimester in human cortical development. And at these early stages the cortex consists of a thin layer of neuroepithelial cells surrounding the ventricle.

So one of the methods that we have used to analyze the proliferating events in the developing cortex is the use of a retrovirus that has been engineered to express the gene for green fluorescent protein and the retroviruses injected as shown here into the ventricle in utero in the embryo, and it infects the cells lining the ventricle and incorporates into the DNA when those cells go through M phase. It is then inherited by all the daughter cells and so it behaves as a clonal marker; it allows us to look at cells, all of which have derived from a single infected precursor cell. So when we look at individual clones after several days, they consist of what is shown here in higher power on the right hand side. Typically, one radial glial cell which spans from the ventricular surface at the bottom to the cortical surface of the pia at the top of this image with a cluster of cells, which we now know are neurons, that are arrayed along that radial fiber. The classic image of the radial glial cell as shown here in serial EM reconstruction of Pasco Rakic's from 30 years ago shows that static function of a scaffolding and a guideway for migrating neurons.

And there is a migrating neuron shown on the right-hand side with a leading process and a trailing process twirled around that radial fiber.

In some of our clonal studies, we have seen very much the same kind of image as shown here on the left. This is shown in segments. So the left-hand image shows the radial glial cell contacting the ventricle; the middle panel shows the radial fiber which makes contact with blood vessels, a characteristic feature of radial glia, where those asterisks are. And then the right-hand panel with the arrow shows the radial fiber with a neuron migrating around looking very much like that serial EM reconstruction of Rakic's; the difference is that these are clonally related cells and so this tells us that the neuron is in effect migrating along the parental radial glial fiber, as it were. Now on the right-hand side there is another clone that consists of many more neurons arrayed along the radial fiber. And I just wanted to point out that they do not all look like these bipolar classic migrating neurons; in fact, most of them look multipolar. And at the end of this talk I want to explain how those are also migrating neurons.

But the first evidence we had some years ago that these radial glial cells were generating neurons was from looking day by day at how these clones change. So on the left-hand side, typically a clone after just a 24-hour infection consisted of just a single radial glial cell. A day later, as shown in the middle set of panels, there was a radial glial cell with one or two neurons associated; and in the right-hand panels at 3 days, radial glial fibers are now grown and there were 3 or 4 neurons associated with the fibers, many of them looking like those bipolar migrating cells. And those are shown by the arrows. So this sequence suggested that the radial glial cells were going through a series of what are known as asymmetrical divisions. So at each division, self renewing as a radial glial cell in generating a daughter neuron. This was confirmed by looking at Sly's cultures over time lapse; and these are time-lapse images from left to right and they show that we could observe the progression of the cell cycle in the radial glial cell and toward the right-hand side of these sequences, the generation of a daughter cell that became a neuron and migrated away.

The next question had to do with whether all the neurons in the developing brain derived from radial glial cells, and this is a question that was asked by a number of investigators. And I just wanted to show one example that we used – we reasoned that we know all the cells that are generating neurons divide at the surface of the ventricle in embryonic ages. And we used an antibody that told us which cells were radial glial cells in that zone, is known as 4A4, and it shows up as red in the upper left panel. So all those red cells are radial glial cells in M phase dividing, and it turns out that all the cells that are dividing in that zone, as shown in the histogram on the right, are radial glial cells in M phase. So that meant that all the progenitors dividing at these ages actually represent radial glial cell divisions. And I just point out in the channel of the 4A4 staining shown in black and white at the bottom, that these M-phase radial glial cells have the characteristic radial fibers that define radial glial cells even while they are going through M phase.

So radial glial cells generate neurons through these asymmetrical stem cell divisions. They are now known as stem cell divisions, because the progenitor self renews and

generates a different type of daughter cell. And I just wanted to show you an example of this in time lapse.

So this is a coronal section that was infected in utero with a retrovirus and then sectioned 24 hours later and cultured, so that we could observe the behavior of this individual progenitor cell. So the red arrowhead points to the radial glial cell, which is progressing through the cell cycle. The white arrowhead is a daughter neuron that was born before we started the imaging. And the surface of the ventricle is at the bottom of the slide, where the dashed white line is and that is where this radial glial endfoot is secured. So the cell nucleus, which is the bulge that you see, drops down to the surface of the ventricle as it progresses through M phase, and the cell rounds up and then divides, never losing that radial fiber. And once the cell divides, shown in the left hand panel in these sequences, the daughter cell now shown with the white arrowhead migrates away from the parent cell, develops processes, and becomes a neuron. The progenitor cell, that is the radial glial cell, goes through another division as shown with the two red arrowheads, so that the initial daughter cell was an asymmetric division as shown here, because one of the cells became a neuron and the other one was a radial glial cell dividing again; clearly, two different asymmetrical fates of the daughter cells.

And as I had mentioned, the radial fibers persist as the cell divides, and this would make sense because as you know, the main function of these radial glial cells in guiding migration requires that the neurons migrate along the radial fiber. And it had traditionally been thought that when progenitor cells like epithelial cells divide, they rounded up. And if these cells, in fact, withdrew their processes and rounded up, they would leave those migrating neurons stranded behind.

And as shown here in cells that are arrayed as they progress through M phase, from A all the way to M, the radial fibers never do go away, even as the cells enter telophase and a cleavage furrow begins to separate the two daughter cells. The radial fiber is more or less committed or inherited by one of the two daughter cells and is never retracted. Now, these are asymmetrical divisions, as I have mentioned; but, one of the principles of asymmetric division relates to the inheritance of intrinsic fate-determining substances that are asymmetrically distributed in the two daughter cells. This is thought to be the basis of an asymmetric division, and it comes from many, many studies done in invertebrates, especially in fruit fly nervous system development.

And one of these intrinsic fate-determining substances is known as mNumb. An antibody staining of mNumb in the cortex, as shown in the left hand panel, and the obvious concentration of mNumb is at the surface of the ventricle, at the bottom of the slide. And that is exactly where these asymmetrical divisions occur and, as shown in the panel in the center, radial glial cells express high concentrations of mNumb right along that ventricular surface. But the mNumb has to be asymmetrically inherited by the daughter cells in order for the two daughters to have different fates. And what is shown here is one of the cells in the stages of telophase where the two daughter cells are beginning to separate, and the middle panel shows mNumb distribution. And the merged panel at the bottom shows a crescent of mNumb inherited by one of the two daughter cells. In this

case, if the daughter cell opposite to the one inheriting the radial fiber, and it may be this asymmetrical inheritance of fate-determining substances that actually dictates whether the cell is going to have the same or, in this case, an asymmetrical fate.

But in developing nervous system, neurons are also born by symmetric as well as asymmetric cell division. Asymmetric cell division is where the two daughter cells have the same fate; but in cortical development, we have observed that the symmetrical divisions occur in a different proliferative zone. And shown here from left to right is a radial glial cell that has generated a daughter cell, the white arrow, that has moved away from the ventricle, rounded up, and divides to produce two daughter cells shown with the two white arrows that then migrate along the radial fiber and turn into neurons, as shown in the right-hand panel. So these progenitor cells are generated themselves by the radial glial; they move away from the ventricle into a zone known as the subventricular zone, and they divide symmetrically to produce a pair of neurons (time-lapse images). And I would like to show you an example of what those divisions look like in, again, time-lapse images.

So the surface of the ventricle is at the bottom of this image, and this cell has moved away, rounded up, and is now in the process of dividing. And when it divides, it produces two neurons that then migrate away to occupy, in fact, the upper cortical layers. So this is a generation of neurons by symmetrical cell division in a different zone than the one where asymmetric neurogenesis occurs. And this another example shown from left to right where the neurons at the right hand side had been recorded at the end of the imaging study with patch clamp electrodes and confirmed to be neurons because they have inward gated sodium channels that are sensitive to TTX as shown in the right-hand side. And they in fact can fire action potentials.

So these are physiologically demonstrated to be neurons. Also when these studies are over, we can fix the tissue and look for marker expression. In this case we looked to see whether they in fact expressed GABA or glutamate; this shows that these pairs of cells generated by symmetrical division are GABA negative. In fact, they eventually become pyramidal neurons in the upper cortical layers. So for many studies of this kind, we made the following conclusions:

That symmetrical and asymmetrical divisions occur during cortical development, but in different zones. Asymmetrical division occurs at the surface of the ventricle as shown here, where about 80% of the cell divisions generate neurons that way. But symmetrical neurogenesis occurs in the subventricular zone, where nearly 100% of the divisions there are symmetrical. And this suggests that there may be epigenetic, that is, a non-cell autonomous signals that might instruct a cell about whether it is going to be born through the symmetrical or asymmetrical division pattern. We will talk about potential signals in just a few minutes.

Once the cells are born, they migrate out of the proliferative zone through a series of phases. And this is another change that I am thinking about how radial migration occurs.

The traditional view as shown by this image again of Rakic's is that neurons once generated hop onto one of these glial fibers and guide their migration up into the cortex. It turns out that cortical migration is a little bit more complicated than that as shown here schematically from left to right. Once a neuron is born at the surface of the ventricle, it rapidly moves away into the zone known as the subventricular zone I referred to before. And it stays there or sojourns in that zone for 24 hours or more. During that time, as shown in Phase 2 of migration, the cells are very dynamic. They extend and retract processes; they can even move laterally. They then often enter Phase 3, where they go retrogradely toward the surface of the ventricle, contact the ventricle, and then rapidly pull in the other fibers and become bipolar. They develop a true polarity then migrate along this gliophilic radial migration shown at the right-hand side as Stage 4 of radial migration. And I wanted to show an example of how this occurs.

This is a single cell that has been generated down at the surface of the ventricle and has then moved to the subventricular zone, where it has been for a day or so, and then moves down toward the surface of the ventricle before suddenly becoming more of a bipolar cell and migrating to its destination in the upper layers of the developing cortex. So these 4 phases of that form of migration, that cell again is shown here and then in the right-hand side, the electrophysiology to confirm that, in fact, it is a neuron and not some form of glial cell.

The symmetrically generated cells. Oh I am sorry, before I do that I just want to mention: circuit formation begins much earlier than had generally been thought. Even before the cell begins radial migration, it becomes polar and starts expressing an axon. As you can see in the panel on the left, the axon, shown with the red arrowheads, begins growing even before the neuron starts radial migration up to the cortex and by the time the neuron reaches its final destination, the axon can even extend subcortically to target structures in the thalamus for example. So early circuit formation is occurring much sooner than had previously been thought.

This is also true for the symmetrically generated neurons that go through a similar 4-phase stage of migration. So this is an example of one of the cells that is dividing in the subventricular zone to produce two neurons. And they begin to extend processes before they start radial migration. And these are, by the way, symmetrical neurons that wind up in the same cortical layers. Now the significance of the different phases of migration that I have emphasized is that we think certain cortical malformations result from the failure of neurons to make the transition between one stage and another.

And this is preliminary data looking at a gene that is responsible for lissencephaly, Miller-Deaker, lissencephaly in humans, this gene has been identified, and what we have done is generated a strategy, an siRNA strategy, to essentially mimic the effect of that gene defect in developing cells. And this construct has been electroporated into just some of the progenitor cells and then 4 days later we look to see how they had migrated. And the green cells shown here at the top of the cortical plate on the right hand side were just control cells that had over the course of four days made it to their appropriate destination in the cortex. Some of the red cells, however, contain different amounts of this gene

defect (siRNA), and the square, which is shown enlarged on the left-hand side, shows a cluster of these cells, and they failed to migrate up into the cortex. In fact, they retained multipolar morphology and have failed to make the transition between that multipolar stage of migration and the radial bipolar stage that is required to get them up into the cortical plate. And so we now have time-lapse images that confirm this and suggest that these cells are unable to make that critical transition.

Now the inhibitory interneurons, as most of you probably know, are not generated into the cortex at all; they are generated in the mesial ganglionic eminence, which is an area of the ventral telencephalon. That is shown schematically here, where the MGE cells, the GABAergic interneurons migrate into the cortex tangentially but once they get to the cortex, they hook up with the radially generated pyramidal cells that we have been talking about so far. Interestingly, they also go through a series of phases as they reach their terminal destination that is shown schematically here on the left hand side. So John Parnavalas and his colleagues have shown that about 70% of the interneurons, when they reach the cortex dive down to the surface of the ventricle, contract the ventricle, and then migrate radially to their appropriate layer. And this is exactly the pattern of migration that I described for the radial migrating pyramidal cells and suggested to us that possibly these two cells are forming connections with each other or receiving some form of similar instruction, possibly telling them which layer to go to. One of those possible instructions could be mediated through GABA. The interneurons as you all know are inhibitory cells that secrete GABA and the radial glial cells themselves, as shown here, express GABA_A receptors. And these are voltage-clamp recordings of radial glial cells from embryos showing that they respond to GABA with big currents. In fact, they have a very high sensitivity to GABA and once GABA is active, it does not desensitize the current so that these receptors are designed in a sense to maximize detection of GABA stimulation.

And some years ago, Joseph Laturco who was a student then in my lab, showed that GABA has the effect of suppressing or regulating neurogenesis, which is shown at the bottom in the histogram. GABA actually in this case inhibits binding and incorporation into progenitor cells. And blocking GABA receptors in the intact brain has the opposite effect – it increases the number of cells that are entering the cell cycle. So GABA can, by regulating receptors on radial glial cells, influence neurogenesis. But the interesting feature is of course that the GABA is now coming from cells that derive from a different area and that come into the cortex. And this suggests that those migrating interneurons might be regulating neurogenesis of the excitatory cells. And, based on what I have described of regulations of divisions within ventricular versus subventricular zone, we now think that the regulation site might be at the point of deciding whether to produce symmetrical or asymmetrical generated daughter cells.

The effect of that would be to increase or decrease the number of cells destined for a specific cortical layer, so that the effects of this kind of interneuronal signaling might be to alter the number of cells that are being born for a given cortical destination. After all the neurons reach the cortex, the radial glial cells themselves disappear and they do so by transforming into astrocytes. And these are examples of some clonally infected or retrovirally infected cells that are turning into astrocytes as shown at the bottom with the

arrowhead. And this is a schematic of how that final cell division occurs. So the green cell is the radial glial cell and when it gives its final cell division, it generates one of these transit-amplifying cells, and the progenitor then moves away from the ventricle, divides symmetrically to produce two neurons. But meanwhile, the radial glial cell loses contact with the ventricle, translocates into the cortex, and becomes an astrocyte. It thus depletes the progenitor population at the end of neurogenesis. But this raises the possibility of what becomes of those adult astrocytes that had during early development the neurogenerating cells. And they may actually retain the potential to generate neurons under certain circumstances. And I am just showing some data from Magdalena Goss's lab, and she has shown on the left-hand panel that radial glial cells while they are making neurons express among other factors a transcription factor known as PAC 6. That is shown in green and overlapping yellow on the left-hand panel. That transcription factor PAC 6 is turned off when the radial glia turned back into astrocytes and stopped making neurons. But she has reintroduced PAC 6 into astrocytes taken from the postnatal brain and that is shown in the panels on the right and in culture has shown that by introducing this transcription factor, she has gotten the astrocytes to start making neurons again. And that is shown in the panel on the right-hand side B and D. So it may be possible to tweak these astrocytes which are distributed through the cortical regions of the brain and get them to make neurons again, possibly under pathological conditions following strokes or seizures for example.

Now the conclusions I just wanted to highlight up to now are first that radial glial cells generate neurons, and they do so either directly through asymmetrical or stem cell divisions or through intermediate precursor cells that divide symmetrically to generate neurons. There are distinct niches that support both symmetrical and asymmetrical neurogenesis and raises the possibility that there are local epigenetic signals that regulate the way these cells divide, and that migration does not occur as was thought traditionally just radially by a single mode, but rather there are distinct phases of migration and that certain neuronal migration disorders, many of them associated with epilepsy, may result from a failure of cells to make a transition between one stage of migration and another.

And then finally, I want to mention the people in my laboratory who have contributed to this work, especially Steve Noctor who is a senior postdoc who did most of those time-lapse films. Thank you for your attention. (Applause)

(Dr. Jeffrey Noebels)

Thank you for that extraordinary framework, and I am thinking that most of you, like me, are wondering how long it will take to look at some of the genes that are now being discovered for migratory defects in epilepsy and to look very precisely at what some of the earliest defects that they can show using these wonderful assay systems. Drilling further into this story of building the cortex and the mistakes that can happen, we need to start thinking about the interneuron populations that Arnie has introduced to us and how they enter the cortex and how they differentiate and connect into the circuitry.

It is a great pleasure to introduce our next speaker, who is a real pioneer in thinking about the molecules that define different regions of the brain. He began some years ago with an antibody that defined the classical limbic system in the brain and has been thinking a lot about other molecular signaling that directs the migration and excitability of inhibitory cells in the brain. So it is a pleasure to introduce Dr. Pat Levitt who is Professor of Pharmacology and Director of the Kennedy Center for Mental Retardation at Vanderbilt, and he will talk about his work and also some fascinating overlap with autism which as you know has an interesting intersection with epilepsy as well.

Pat.

(Dr. Pat Levitt)

I am going to turn your computer down, Arnold, because two screens at the same time are tough for me to concentrate on. Can you hear me with this? Yes, you are okay? Okay.

So, I do want to start by pointing out that Jeff Noebels is as dedicated an individual as I have ever met and has a passion for a number of things including this society, because last year at this time, their meeting was in Boston and I was due to speak at another symposium and Jeff had me on the phone Friday afternoon. I think the symposium was Sunday, and he was trying to convince me that if I started Friday morning from Nashville, I could actually walk to Boston and make it in time for the Klingenstein Symposium. So Jeff, thanks for keeping me in mind and inviting me for this wonderful occasion.

So I am going to talk about topics that relate to what we currently know about how the other class of neurons that Arnold touched upon, the interneuron population, are added to the developing cortical circuitry and trying to keep in mind and put in perspective the idea that the balance of excitation and inhibition in large part really depends upon how well the nervous system, how well the forebrain, controls the formation of the interneuron population as well as regulating its differentiation. And I think Jeff alluded to one of the principal problems that we have in trying to understand this, because as we know, there are a number of genes and molecules that are probably responsible for directing the initial development of this and other critical neuronal populations in the cortex, but at the same time we understand that experience and activity plays a crucial role in driving the further differentiation of this cell population. So how do we make sense out of whether we are looking at a chicken or an egg in the context of pathophysiology? So what I am going to talk about is how the interneuron and its development may be one point of convergence in a number of different clinical neurodevelopmental disorders that have at its heart problems in information processing and problems in the balance of excitation and inhibition. I am going to talk about some of the neurodevelopmental mechanisms that we understand now in terms of what is regulating this cell population. I am going to talk about the fact that we now know that these interneurons do not develop en masse in terms of having a single mechanism of formation, but rather I am going to show you in an animal model evidence that you can actually disrupt different subpopulations of interneurons that may be caused by a single

gene mutation. So while some interneuron populations in the cortex are left intact, others may be highly disrupted. And not only can there be selective disruption of subtypes of interneurons, but there is evidence now that this can be disrupted in a regional fashion so that some parts of cortex may have their circuits intact, other parts of cortex actually may be quite disrupted. And that when you put this together, you end up with a rather complicated pathophysiology with more than a single domain that may be disrupted at a functional level, and I am going to show you some of the work that we have been doing in a mutant mouse to show you that, both behaviorally and in terms of excitation inhibition balance, there are major changes that occur. Okay.

So in some sense, most of us are stuck in this condition in which we understand the clinical syndrome quite well, and we use descriptive approaches to understand the pathophysiology. And I think it is fair to say that we have been very good at describing the changes that have occurred in a particular clinical syndrome. The real challenge and the reason why many of us are focused on trying to translate information that we understand from the clinical syndrome and go back into experimental conditions to try to get at key issues is to try to get at the pathogenesis, try to understand how that pathophysiology arose in the first place because we believe, I think as a group, that if we understand the pathogenesis of a disorder, prevention will not be far behind. And I have already alluded to the fact that my own bias is to think about how the interneuron itself may be a point of convergence, in fact a point of vulnerability for a number of neurodevelopmental disorders. Now the reason I say this is because there is actually a lot of functional evidence that the interneuron is really critical in a number of different aspects of cortical development and cortical function.

I have two of them shown here. One is that the interneuron is responsible for regulating the precision in which a pyramidal cell, a projection cell, actually reads the thousands of inputs that are coming into it over a period of time. How precise is that pyramidal cell able to decipher all those thousands of bits of information? It is the interneuron that is essentially setting the timeframe, setting the filter window in a sense, in order to be able to have that projection neuron understand the kind of information that is coming in. The other thing that the interneuron is critical for, in terms of adult function, is to regulate the output. It regulates the precision at which multiple populations or projection neurons actually fire as a group to send information to another location. So these are obviously two key areas in which the interneuron is responsible for precision and regulation, regulating how the cells interpret input and regulating how well the cells then send information out to other cortical areas or subcortically as well.

Now what is remarkable about the interneuron and its relationship with the projection neuron is that when you look across mammalian species, all mammalian species that have a 6-layer cortex, the ratio of glutamatergic to GABAergic cells, the ratios of excitation to inhibition, is essentially constant; not just across all cortical areas, but across species. So this ratio is about 6:1; some say 5:1; some say 7:1; but essentially, people agree that this is one of the components of cortical circuitry that is highly conserved, which suggests two things: Number 1, it suggests that the mechanisms that are in place to regulate the development of the circuitry and to establish this ratio of 6:1 is probably highly

conservative across species as well. And the second thing to keep in mind is that if the interneuron makes up 1 out of every 6 neurons in the cortex and if you add glia into that, which are probably in humans twice as numerous as neurons themselves, any change to the population of interneurons is unlikely to be seen by standard neuropathological examination. And I am going to show you, in fact, a mouse model that you can eliminate 50% of the interneurons and the cortex basically looks the same. And it makes sense in terms of the numbers.

Now, Arnold already alluded to the fact that we have two different domains of the developing forebrain, the gray area that you see there. This is kind of awkward because I am used to pointing at the screen; I am going to walk you through this with descriptive language. The gray area is known as the dorsal pallium – that is the region that gives rise to the cerebral cortex in all mammalian species, and the region that you see below in this diagram that has the descriptors LGE and MGE, that is the region known as the subpallium, which previously we had thought gave rise only to the basal ganglia structures, the striatum and the globus pallidus and other associated motor structures. And Arnold again alluded to this, and I can move through this rather quickly, that dogma up until a few years ago was that the dorsal pallium, the ventricular zone, the region that contains the progenitor cells gave rise to all of the neurons that ended up in the cortex, both the projection neurons and the interneurons. Well we know that is not the case now, and I just want to go through a few experiments that were not from my laboratory that really are classical and which these individuals deserve a lot of credit for changing the way that we think fundamentally about how this cortical circuitry forms.

There was a study that was done by Juan DeCarlos in Frank Valverti's lab in 1996 in which he took a piece of dye and what you see there is a drawn pipette filled with the red dye in cartoon, stuck into that ventral part of the telencephalon, the subpallium that was thought to give rise only to the basal ganglia. And they were trying to track projections that might go through there from the cortex, neurons in the cortex that would project down through the internal capsule, and they did this at a time when they thought the axons were just beginning to emerge, so what you are looking at in that large section, that dark field section, a blob of red is the dye that has been stuck into the ganglionic eminence. And instead of seeing axons that could be traced back from the point of insertion of the dye up into the cortex, you can see those white arrows are pointing to cell bodies that are sitting in the neocortex. Well that was the first clue; that was the first evidence that in fact cells that were originating down where the dye was inserted, down in the forerunner of the basal ganglia, actually was giving rise to cells that were migrating rather long distances up to the dorsal pallium.

Now Stuart Anderson and John Rubenstein's laboratory in 1997 did a seminal study in which they were able to demonstrate in mouse models in which they looked at animals that carry mutations (I am going to have to look here), animals that were carrying mutations for genes that were responsible for regulating the development of the ganglionic eminence. They were able to show that if they disrupt those genes, disrupt the development of the ganglionic eminence all of the interneuron populations that should have ended up in the cortex were in fact absent. And you can see on the left side where it

says +-+ that is what the normal pattern of developing interneurons looks like; the cell staining is for GABA. And if you look on the right side where you can see the -/-, that is the mutant mouse, and you can see that the NCX, the neocortex, is virtually devoid of interneurons. This is really the first experimental evidence that shows that if you disrupt the basal forebrain, the part of the brain that should give rise to the basal ganglia, you also disrupt the formation of the interneuron populations.

Now it has been suggested, and there is pretty good experimental evidence for this, that in fact there are major domains of formation of different cell populations based on their neurotransmitter. So that the dorsal pallium, which is shown here in purple, gives rise to essentially all the glutamatergic cells that will populate all different parts of the forebrain, whether it is in the cortex or below the cortex. The green region, that is the ganglionic eminence, gives rise to all the interneurons, all the GABAergic cells. And the blue region that is designated AEP/POA, it gives rise to all the cholinergic cells of the forebrain. Now one of the interesting things about this and one of the conundrums we are in in terms of trying to understand how this development takes place and what regulates it is that if you were a GABAergic cell. And you form out of this region, this domain of the ganglionic eminence. And you end up going and migrating long distances up to the cortex, you will become a short axon interneuron that will be intercalated into the local circuitry of the cortex. If however you are an interneuron, you are a GABAergic cell and you end up staying down in the basal ganglia, you will end up using GABA as a neurotransmitter, but you will phenotypically be different in terms of what you function as, because you will become a long projection neuron that projects to other parts of the basal ganglia such as the substantia nigra. So where you end up, like your house in a particular neighborhood, can in some ways really define quite specifically the kind of function that you are going to perform.

Now we know that there are a number of steps that are involved in the regulation of GABAergic production, I have outlined them here, and keep in mind that the more steps we add in development, and Arnold touched on this as well, the more steps that we add, the more places where disruption can take place in terms of altering the molecules that are responsible and even the activity that may be responsible for driving some of these processes. So we first have to specify these progenitor cell populations that is designated 1. We have to regulate whether the cells are actually going to migrate out of the zone or stay in the basal ganglia, become long projection neurons or move up into the cortex. Well we have to regulate whether they are going to migrate all the way up dorsally to the cortex; how they are going to invade into the cortex and intercalate into the circuitry; and then finally, we have to regulate how they are going to differentiate, because we know that there are many different types of subpopulations of interneurons.

Now to go back for a second, I have listed here, and it is color coded on the left, a number of the molecules that we have already identified that we know are important in this process and like many other aspects of development, the same molecule may be used multiple times over the course of development to regulate different functions. And you can see, for example, there are growth factors listed here, BD&F for example, which we

know is important in synaptic plasticity also is important in probably regulating some of this movement.

One of the molecules that I am going to talk about very briefly now that we worked on in the laboratory is hepatocyte growth factor HGF. We stumbled upon it quite accidentally in terms of its role in the brain, but yes, it is the same growth factor, the same HGF that is responsible for the differentiation of hepatocytes, and it is also known as scatter factor, which is involved in regulating the development of lungs. So we did an experiment. And you can see that on top in which we took a piece of ganglionic eminence tissue and put it in a culture dish and over a period of a few days. You may be able to see on the left side where it says control some green cells that look like they are emerging slightly from that X plane, and that is evidence that there is some migration out of that piece of tissue. But if we add hepatocyte growth factor, you can see this rather large halo of cells that has emerged that has been stimulated by the addition of this growth factor. And we did this biochemistry to show in fact that in the brain, at the same time that HGF is expressed in the liver, it is also expressed in these parts of the forebrain that would be responsible for regulating movement of GABAergic cells.

Now I have to just introduce to you how this signaling system works. Hepatocyte growth factor, which is shown on the right hand side of this diagram (up here) is actually formed as a progrowth factor. It is formed as a propeptide, and in order for it to be biologically active, it needs to be cleaved. It needs to be cut in the extracellular region once it is released from the cell. And there are a number of different enzymes that are responsible for this; one of which is known as urokinase plasminogen activator.

Urokinase plasminogen activator is found in the brain; it binds to a receptor known as uPAR and when that interaction takes place, it cleaves. It is most effective in cleaving hepatocyte growth factor, which will then allow it to bind to its receptor C-Met and then a number of different actions can take place.

Now we showed experimentally in those slice cultures again that I showed you before that if you stick a piece of dye into the ganglionic eminence shown here on the left, and you can see the white arrows to display the cells, the interneurons that have migrated up into the cortex, we can disrupt hepatocyte growth factor expression in these slice cultures, and we can alter the ability of those cells to move up into the cortex proper. Now that is in a culture dish, and my laboratory really tries to focus on taking what we find experimentally in a culture dish and see whether we can replicate that or see what happens when we look at the disruption of some of these molecular signaling systems in an animal model. Now of course the problem here is that we are looking at hepatocyte growth factor and its receptor C-Met, both of which are important in placental development, lung development, liver development. And there are mouse knockouts for these genes, but they end up dying prenatally well before we would be able to study interneuron development. So we took advantage of the fact that we have some understanding of the way that this signaling system works. And we looked at an animal that was carrying a mutation for the enzyme and receptor system that were responsible

for activating hepatocyte growth factor, which we call the uPAR, uPAR Met. So that is what I am going to describe now.

Now I am not going to go through the biochemistry, but essentially what we were able to show in this mutant mouse that does not directly cause a mutation in hepatocyte growth factor or its receptor, nonetheless, results in a decrease in expression of this growth factor that we know is responsible in part for interneuron migration. So we were able to show that biochemically there is a disruption of hepatocyte growth factor and its signaling system. Now before we decided to go into all sorts of neuropathological descriptions of what might be going on in this mouse, we wanted to actually determine. We made a prediction. If hepatocyte growth factor signaling was disrupted, it might mean that interneuron development was disrupted in these mice. These mice are viable: they mate; and they seem to behave fine. Although I will tell you that we did see some evidence for changes physiologically in the brains that I will describe in a moment. But we made a prediction that these mice would be more sensitive to seizure inducement by using a conventional experimental model in which we administered PTZ (pentylenetetrazole) to the mouse, and what you see here on the left in terms of severity score of induced seizures, the wild type or normal animals are indicated by the white bars, and the black bars are the mutant mice. You can see that almost 100% of the mice, when injected with PTZ, go into Stage 3 – tonic extension seizure. And a large number of these animals actually died. So with PTZ administration, we were able to show that these mice show an increased susceptibility to induced seizures.

Now that gave us hope and actually stimulated our decision to go in and do what all of you know is painstaking and very time consuming: neuroanatomical analysis. So the first thing we did is we focused on developmental periods embryonically in the mouse and just at birth when these interneurons begin to emerge. And what you see on the left panel where the +/+ is, that designates the normal mice. And you can see the red dots represent interneurons that are developing in the cortex, embryonic day 16.5, postnatal day 0. And the blue sections on the bottom are just counterstained with the dye so we can see the overall tissue morphology. On the right side, you can see -/-, that is the uPAR knockout mouse and you can see that there is a significant reduction in the number of red cells, the number of interneurons that ended up migrating to the cortex in these mice. And I want to emphasize, and Jeff asked me to emphasize this, that if you look at the structure of the cortex, it looks normal. There is normal lamination patterns; we actually went in and counted the number of cells and a number of cells actually, given the variations that one has in counting millions of cells, looks essentially normal. So the structure of the cortex even microscopically, just counting cells generically looks normal. But when we went in and used specific markers for interneurons, we found that there were selective changes. First regionally, I'll show you, up on the top you can see cingulate cortex, parietal cortex, that is the frontal lobe and the parietal lobe where sensory cortex is located, there is about a 50% reduction in the number of GABAergic cells in this mass – 50%. If we look however at other cortical regions in the same brain, pyriform cortex, which is olfactory cortex in the mouse, and visual cortex, that is the most occipital regions, the number of interneurons are in fact normal in this mouse. So single gene mutation changes selectively in frontal and parietal regions, but no changes in occipital.

And this is shown diagrammatically here on the left. You can see that I have diagrammed both the GABAergic cell populations, and I have given away the next slide. We looked at subpopulations of interneurons based on the callosine binding proteins that they express including parvalbumin and calretinin and statin and I am going to show you that data now. The very left histogram shows you in blue the counts of interneurons, the GABAergic cells in the wild type animal and the red is the uPAR knockout; there is a 50% reduction in parietal cortex. The calretinin cell populations are absolutely normal in this mouse, but the parvalbumin cell populations in fact are almost completely eliminated in this mouse. So you can account for the 50% decrease in GABAergic cells by a reduction in almost all of the parvalbumin cell population. In the mouse, parvalbumin makes up about 50% of the subpopulation of GABAergic cells.

One of the things that is not highly conserved across species are the ratios of different subpopulations of GABAergic cells. So in the human for example, parvalbumin makes up about 20 to 25% of the interneuron population, and when you look across species, you see lots of variations in these subpopulations which reflect physiological properties as well as the morphological connectivity.

So in summary, what I have shown you thus far is that hepatocyte growth factor and the uPAR signaling system is involved in the migration of GABAergic cells that we can show that with a single gene mutation, there is an alteration, in interneurons selectively regionally in the single gene mutation. And then finally I have shown you that you can actually alter subpopulations and that suggests that there are different mechanisms for regulating the final development in differentiation of different subpopulations of interneurons; not that I wanted to make the story any more complicated than it is, but there you go.

Now what are the functional consequences of this reduction? We had for the first time an animal that survived with 50% of its interneurons in certain cortical areas reduced. So I turn to the maestro of mouse phenotyping, Jeff Noebels; we sent him these mice and sure enough, these mice exhibit rather unique patterns of spontaneous seizures, which are shown here (and you can talk to Jeff at the coffee break, which will be happening very shortly). So what we saw in terms of induced seizure sensitivity was paralleled by spontaneous seizures that occurred in these mice. The other thing is that we know that interneurons in the cortex and subcortically are in fact involved in a number of different functions. I already told you that they are involved in sort of the general regulation of information processing, information flow, and so we looked at a number of different behaviors in this mouse, one of which was to look to see whether there were changes in anxiety. And I show you on the left an elevated plus maze picture for those of you who do not do mouse behavior, and essentially what we do is monitor how willing the mouse is to go out onto those open arms. This is just a cross maze with walls on one side and open arms on the other and if the mouse goes out onto the open arms, it is like those people who are willing to go up on a skyscraper and walk on steel beams. Mice typically do not like doing this very much; and if you look at the histogram you can see that in the uPAR knockout, which is designated in red and you look at where it says open arm, they

spend almost no time in the open arm; they spend almost all their time either in the center or in the closed arm, indicating that not only did they have spontaneous seizures, but they also had increased anxiety.

We actually tried to intervene and see whether we could ameliorate the anxiety symptoms, but in fact, what we found was by administering diazepam, and we did a dose response. These mice show highly increased sensitivity to diazepam. And essentially what we did is reduce motor activity globally in these mice suggesting that the changes in interneuron development resulted in compensatory changes in GABAergic receptors, something that we would predict based on alterations of the circuitry. Now the other thing that we looked at, another function, because we were interested in GABAergic cells from the perspective of neurodevelopmental disorders, and I will get to that in a moment when I wrap up. But we looked at social interaction in these mice as well in which we had a resident and an intruder. We put a mouse in as the intruder, and we just monitored how these mice interact with each other. And the bottom line in which you can see here in the histogram on the bottom is that these mice interact less with congenic strains, with their own species. They are not more aggressive; they just basically go about their business in the cage and do not interact. And if this profile is beginning to sound familiar in terms of neurodevelopmental disorders (I think I am going to go through this and get right here), it sounds like neurodevelopmental disorders that have a behavioral component, an information processing component, and even a seizure component, and that is autism.

Now I am going to tell you that there is neuropathological evidence that interneurons are disrupted in autism and the best evidence comes from work that Manny Casanova has done, a neuropathologist, in which he has shown that the interneuron development is altered and changes the fundamental structure of the information processing unit in the cortex known as the minicolumn, which is designated here. All a minicolumn is, is a representation of the radial organization of the cortex in which information comes up from the thalamus and flows in a columnar organization up into more superficial layers in which information is integrated and then sent to other cortical areas. And we know from the work of Mountcastle and others that the interneurons, particularly those that are located superficially, are responsible in part for the integrity of this minicolumn, so we have neuropathological evidence both in dyslexia and in autism that this minicolumn is disrupted and that the interneuron might be responsible for that disruption.

Now one of the things when you look at autism and when you look at the constellation of phenotypes, which I have listed here, is that in autism, depending upon which paper you read, there is an estimate of about 15% to 25% of children with autism have overt seizures. There is a very high penetrance of abnormal EEG, also a very high penetrance of disrupted sleep, sleep cycles, and also REM sleep in children with autism. There are obviously changes in social relatedness and anxiety, all of which point again to the possibility that alterations in interneuron development are the cause for vulnerability in a very complex clinical terrain.

Now I am going to summarize now and suggest that the interneuron in a number of neurodevelopmental disorders, not just those that are involved in alterations and excitation may be a point of convergence, a point of vulnerability, I have listed here and I have already highlighted again, and I want to emphasize that this change that we see is selective, both anatomically and in terms of subtype, and in fact depending upon the combination of changes that might occur in terms of different subpopulations, one could imagine a complex pathophysiology or what we would term comorbidity might emerge in a number of neurodevelopmental disorders. I just want to take a second to highlight one other factor.

In our experimental system, when we change the genetic background of the mouse strain, when we move from one mouse strain to another in terms of genetic background, we can change some of the fundamental phenotypes that we see in this mouse. We see a core set of disruptions of the interneuron development, but we see other features that are not maintained on different genetic backgrounds, again highlighting the complexity that we are dealing with in terms of understanding how gene mutations and modifiers of those gene mutations couple with experience and physiological activity to generate a very complex pathophysiology.

I want to recognize Elizabeth Powell who is a post-doc who did most of the work that I talked about in my own laboratories, now at the University of Maryland. A number of people in the laboratory also were involved. And the work that I talked about in my laboratory was supported by the National Institute of Mental Health, the National Institute of Child Health and Development. Have a good coffee break. Thank you. (Applause)

(Dr. Jeffrey Noebels)

Thank you, Pat. Now we all know where interneurons come from. We also know where enthusiasm comes from, from Pat Levitt. Well, it has been announced. There was also a question put before you which is – which comes first, the chicken or the egg? And if you come back at 10:30 sharp, I will tell you the answer. **(Break)**

(Return from Break)-

I think we should go ahead and get started, and I promised I would answer the question of which came first, the chicken or the egg. It depends on who you ask. If you ask the chicken, they always claim that they came first. If you ask the egg, they say well the chicken is just there for me to make another egg. The real point of the story is that the egg that comes before the chicken is not the same egg as the one that comes after and that is what the introduction to our next speaker is all about. It is plasticity, and that it really matters serially what the order of a lot of events is, that they each have effects on the next. And our next speaker, Amy Brooks-Kayal, who is a Pediatric Neurologist at the University of Pennsylvania Children's Hospital, has really spent a great deal of time with her laboratory and her team looking precisely at changes in plasticity in the developing brain at the molecular level. So she is going to be telling us more about the changes that occur in heteromeric complexes that we know and love – ion channels and ligand-gated

receptors and bring electrophysiology of the interneuron and other cells back into the picture now that we have learned so much exciting new biology.

Amy.

(Dr. Amy Brooks-Kayal)

Can everyone hear me without the second microphone? Okay.

I would like to talk to you today about a question that I find myself asking a lot in my career as a pediatric neurologist is, why do children seize so much? This is a question that comes up a lot in the emergency room, in my clinic, and even in general pediatricians' offices and that is really the question, in a way, that Jeff has asked me to address today. So we know on a more formal level that in fact the immature brain is highly susceptible to seizure activity, and we can see this both in the clinic as we all do day to day, as well as in epidemiologic studies that clearly show evidence that the highest incidence of seizures occurs in the first decade of life, particularly in that first year of life when the human brain seems to be uniquely susceptible to seizure activity. In addition, in the laboratory, we find that no matter what animal model we look at, there is really an enhanced susceptibility to any kind of induced seizure activity. In addition, on the other side of the chicken and egg question is, what do those seizures do to the brain? Should we be concerned about seizures in the developing brain or not? And I think that the evidence that I will show you, and evidence that has been accrued by a variety of clinical and basic science studies, is the answer to that is yes. There is clear evidence from both epidemiologic studies in humans as well as animal studies that early life seizures, particularly prolonged seizures and recurrent seizures, really can impact on the brain in a long-term fashion that can enhance a susceptibility for later development of epilepsy.

So, specifically the questions that Jeff asked me to address today are three. The first is, what specific differences in the neurotransmitter systems present in the immature brain may increase, may contribute to this increased seizure susceptibility that we have all witnessed. Secondly, when seizures do occur in the very young brain, do they in fact then alter normal activity-dependent patterns of development, which basic developmental neuroscience has shown over the last decade to be a predominant form of plasticity early in development. And finally, a question that I will make allusion to that answer at this point in time is, can we use these unique molecular alterations that occur after seizures in the developing brain as therapeutic targets for hopefully the prevention or cure of epilepsy after early life brain insults.

So, I kid my fellows in the laboratory that I went into epilepsy research; because it is easy; because it is always one of two things. It is either too little inhibition or too much excitation, and I could handle that as a pediatrician. This was something I could wrap my head around. So and in fact in the developing brain, the answer is, it is both things; there is both relatively slow maturation of inhibitory receptors and there is also rapid and even exuberant maturation of the excitatory neurotransmitter system. And the combination of

these two factors really contribute to an imbalance of excitation and inhibition in the developing brain.

So, we are going to start on the inhibitory side of things because that is where a lot of my work in the laboratory has been and as you are all aware, gamma-aminobutyric acid or GABA is the main inhibitory neurotransmitter system in mature neurons. And I will come back to that caveat in that moment. There are three receptors, primary receptor subtypes, GABA_A, B and C receptors. GABA_C receptors you will not hear much more about today, because they are primarily retinally localized and are thought to be less important in epilepsy obviously for that reason, except maybe retinal kindling. And the GABAergic system in general has been of much interest to epileptologists for decades, because we know that many of the drugs that we commonly use work on this system. So by gabatrin and tiagabine both act by different mechanisms to enhance synaptic GABA levels and a variety of commonly used AEDs work directly by augmenting GABA_A receptor activity, including phenobarbital, benzodiazepines and to a lesser degree, valproate, topiramate and levetiracetam.

So GABA_A receptors mediate the majority of the fast synaptic inhibition in the brain and are postsynaptically localized. They are anion-selective and gate primarily chloride; although, under certain circumstances can also gate bicarbonate. They are actually not single proteins, but rather a complex of multiple protein subunits. And there are a variety of different families, which you see projected on the slide – alpha, beta, gamma being the 3 large families and also some other ancillary subunits. And each of those families has a variety of different subtypes. The importance of this is those different flavors of receptor subunits or different subtypes can give dramatically different properties to the receptor. So if you take a receptor with two α subunits, two β subunits and a γ subunit, which is thought to be the most common composition in the brain, and you take one of those α subunits and you change it from an $\alpha 1$, which is the most highly expressed in the mature brain, and you make it an $\alpha 4$, you take the receptor from being very sensitive to augmentation by benzodiazepines and turn it into a receptor that is essentially insensitive to augmentation by benzodiazepines. Obviously, something that could have dramatic effects on our ability to use benzodiazepines to treat seizures.

In addition to modulation by antiepileptic drugs, GABA_A receptors are also the site of action for many general anesthetics, neurosteroids, and are also modulated by the endogenous ligand zinc. GABA_B receptors are responsible for the slower, more long-lasting inhibitory currents. And there are metabotropic receptors that are linked to G proteins. They are localized both pre- and postsynaptically and like GABA_A receptors, they are heterodimers, which means that they are composed of multiple subunits – primarily R1 and R2, which have additional diversity that is added by splice variance. And again, like GABA_A receptors, GABA_B receptors are widely distributed in the central nervous system, particularly in the hippocampus, cerebellum, and thalamus. Now the GABAergic system, as I mentioned, provides the majority of inhibition in the mature brain; however, things are entirely different in the immature brain. What we know is that unlike interneurons where opening a GABA_A receptor channel results in neuronal hyperpolarization and inhibition of cell firing in immature neurons, GABA_A receptor

activation is actually excitatory and results in membrane depolarization. And a variety of laboratories, including Dr. Ben-Ari's laboratory, Dr. Kriegstein's laboratory with some data you heard about earlier, and others have shown that these depolarizing GABA currents are actually critical for a whole variety of normal developmental processes, including, as we heard about, neuronal proliferation as well as migration targeting in synaptogenesis.

So, how does this work? How does this wholesale switch occur from excitation to inhibition in the developing brain? Well, as it turns out, it really has little to do with intrinsic properties of the GABA_A receptor itself. Rather, it seems to be dependent on changes in the chloride gradient that occurred during the course of development. So in mature neurons, which we are all familiar with, we all remember from our neurochemistry classes that the chloride concentration in the cell is quite low. This low chloride concentration is maintained by the presence of a large number of chloride extruding transporters called KCC2. So since chloride is low inside the cell, when GABA is released, chloride flows in along its concentration gradient and you get membrane hyperpolarization, which again, just as we had all learned back in our introductory neurophysiology courses. Things are very different, however, in the immature brain, where there is actually very low expression of the KCC2 chloride extruding transporter during the first 2 postnatal weeks in rodent and probably, we do not know this exactly, probably through at least around the time of term in human, based on homology between rodent development and human development. There is, however, expression of the NKCC1 transporter, which actually brings chloride into the neurons. So what you have is chlorides that have a much higher intracellular chloride concentration early in development. Well, not surprisingly, when GABA channels are open, chloride then flows along its concentration gradient, which is now in the opposite direction, and you end up with cellular depolarization. Again, a profound wholesale switch in the activity of this receptor early in development.

We know, as I mentioned, that these currents, these excitatory GABA currents, are critical for normal developmental processes. And there is also increasing evidence that they may also play a critical role in ictal activity in the developing brain. So it has been known for a while from working Dr. Ben-Ari's laboratory and others that synchronous neuronal activity in the hippocampus can be driven by GABA_A receptors and blocked by GABA receptor antagonists. More recent evidence from both Dr. Ben-Ari's laboratory and Kevin Staley's laboratory suggests that more directly this GABA mediated excitatory activity may actually be critical for ictal events in the developing hippocampus. So this is in vitro work from Kevin Staley's laboratory, where he looked at immature hippocampal slice taken from postnatal day 11 to 12 animals. Again, in rodent, that rodent period is approximately equal to term or the early neonatal period in human to the best of our ability to make that association. And what he finds in that immature slice is that high potassium induces ictal activity, ictal like epileptiform activity. Now interestingly, when he uses a GABA_A receptor blocker, which in mature neurons would normally worsen that activity, he can actually block that ictal-like activity entirely. And in turn when he uses something that activates GABA_A receptors like muscimol, he actually sees an increase in

frequency of that activity, again suggesting that GABA-mediated excitation is critical to producing these ictal discharges.

This is work from Dr. Ben-Ari's laboratory and slides that he was kind enough to send me looking at another in vitro system, which is the interconnected hippocampal slice system. And in this system, the two hippocampi from an immature postnatal day 7 animal are kept in independent chambers, so they can be independently perfused with agents. And what they found in work with Dr. Khalilov and Holmes with Dr. Ben-Ari is that when you applied kainic acid to one of the hippocampi, initially those discharges are propagated to the opposite hippocampi. So you can see here on top the ipsilateral hippocampus and the propagation of those discharges to the contralateral hippocampus through the remaining commissural fibers that connected to hippocampi. Now initially, these discharges are synchronized but if you apply kainic acid repetitively about 10 to 12 times, and then you pharmacologically separate the two hippocampi using tetrodotoxin (TTX), what you find is that contralateral hippocampus, which has never seen kainic acid, becomes independently capable of generating ictal discharges or the plastic mirror focus that all we clinicians always worry about in epilepsy.

They went on to look at what was mediating these independent ictal discharges in the mirror focus and found in fact similar to what Kevin saw in his work, that GABA receptor blockade with bicuculine actually stopped these discharges in the epileptic slice. And they further went on to use perforant patch recording to look at the chloride reversal potential, confirming that GABA was excitatory in these neurons, again strongly indicating that GABA-mediated excitation in the developing hippocampus can underlie ictal activity. Well at the same time and a little bit later in development, you see that there are changes in the intrinsic properties of the receptor as well as changes in the chloride gradient.

This is an NC2 hybridization study that was done by Peter Seaberg and colleagues, now over a decade ago, just to show you the profound changes in one of the 14 different GABA_A receptor subunits during the course of development. This is the $\alpha 1$ subunit which you will hear more about later because it is the predominant α subunit in the forebrain in mature animals. But as you can see, in contrast to the rather ubiquitous expression, which you can see all the light stuff there is GABA_A receptor mRNA, $\alpha 1$ mRNA, there is really very low levels of expression in the immature brain. We looked at this when I first started foraging into the GABA field now. Sadly also over a decade ago, I did a postdoc in Dolan Pritcher's laboratory at University of Pennsylvania. And I was interested as a pediatrician who had not even done my neurology training yet, and whether something similar to this was happening in the human brain. Because you know, the rodents were cute, but I wanted to know what was going on in my patients. So, we looked at expression using mRNA protection assays in both cortex and cerebellum of the GABA_A receptor $\alpha 1$ mRNA over the course of human development. Our earliest time point was 36 weeks postconception, and we looked up through adulthood. And what we found was over a 3-fold increase both in cortex and independently in cerebellum in $\alpha 1$ subunit expression. And we also did pharmacological studies that confirmed that the

functional protein was being inserted into the receptor. And the receptors showed more of the characteristics of containing $\alpha 1$ as these neurons matured.

So, a variety of laboratories from all over the world have studied different aspects of GABA_A receptor expression and function over the course of development and generally what people have found is that there are clear regional differences in subunit expression and how subunits change over the course of development. There are a few overarching patterns though. Some certain subunits tend to show a higher postnatal expression and then either stabilize or decline such as the $\alpha 2$ and $\alpha 3$ and $\alpha 5$ subunits, while others, such as that $\alpha 1$ subunit that I mentioned before and the $\gamma 2$ subunit seemed to progressively go up over the course of postnatal development. And coincident with these changes temporally, other laboratories have seen significant changes in the function in pharmacology of the GABA_A receptor. So over the course of development, the receptor kinetics become more rapid. There is increased sensitivity to benzodiazepines, and there seems to be decreased sensitivity to inhibition by zinc.

So our laboratory was interested in whether similar changes were happening at the level of individual neurons. And I was very fortunate to be able to collaborate with my colleague Dr. Doug Coulter, also at University of Pennsylvania, to look at this question with an individual dentate granule neurons in the hippocampus. Now when you go from billions of neurons to a very small subset, you might ask, well, why dentate? Why did you pick those neurons? One reason is, is that they are the latest developing of the hippocampal principal cells. So if you are interesting in looking at postnatal development, those are the cells really that continue to change during those first few postnatal weeks. The second reason is because of the important role that we all know of the epileptologist of the dentate gate and the importance of dentate inhibition in controlling activity and turning into the hippocampus. So for those reasons, we looked at individual dentate granule cells isolated from animals beginning in the first postnatal week through adulthood. And members of Doug's laboratory, including Melissa Schumate, one of his students, logged physiologic recordings on the cells characterize their GABA currents, and then our laboratory amplified their RNA to look at subunit expression. And what we found is that as we saw at a regional level, there are very specific changes in subunit expression that occur within individual neurons over the course of early postnatal development. So from the first postnatal week through adulthood, there is overall a greater than 2-fold increase in subunit expression within the individual neurons and this happens in particular subunits such as the $\alpha 1$, $\beta 1$ and $\gamma 2$ subunits that seemed to increase most significantly. And in these same neurons, the physiology of the receptor was changing as we would expect with an increase in GABA current density, more augmentation of zolpidem in mature neurons, and less inhibition by zinc in mature neurons.

So overall, there are profound differences both at a regional and an individual neuronal level in GABA_A receptor activity over the course of development. With immature neurons really having fewer GABA receptors, lower GABA-mediated currents, and less augmentation by benzodiazepine. Now for the clinicians in the audience, you are probably going, she has talked about a bunch of Greek letters, and I have no idea why

this is important to me. Well, think for a minute back to what I said initially about GABA receptors. You guys use these every day; I use them every day when I am in the clinic, because the agent that we use most frequently to treat neonatal seizures act on this receptor, right – phenobarbital and benzodiazepines. When I am called to the NICU to see a seizing infant, I say give him Ativan. And when that does not work, load him with phenobarbital. Well, I just told you that this system is uniquely immature and not only is it at times excitatory doing exactly the opposite of what we want, when it matures a little more, it may be inhibitory, but it is very hypofunctional and really not prepared to be sensitive to the medicines that we are using. So this really does impact on our pharmacology and our treatment of our patients on a daily basis.

What about GABA_B receptors? Are they positioned to sort of take up the slack in the inhibitory system? And as you might guess, the answer is no. There is some early expression of the presynaptic GABA_B receptor subunit GABA_{B1A} at birth; however, the postsynaptic GABA_B subunit has very low levels of expression in the first two postnatal weeks and then progressively increases. And functionally, you see exactly what you would expect in association with this, which is, there is some presynaptic GABA_B receptor function in the first couple postnatal weeks, but almost no postsynaptic GABA_B receptor function until the second or third postnatal week, again suggesting that the levels of inhibition from both the GABA_A and the GABA_B receptors are very low during this early neonatal time period. Well what about the other side? As I said, you know, epilepsy always has its two sides. What about the excitatory system? Well at the same time that the inhibitory system is very slowly developing, the excitatory system is like the rabbit and the hare; it is way out in front, and actually as you will see, has an exuberant period where it overshoots the levels of adult excitation. So this is for those of you that are microbiologists can nap for the next slide. Those of you that are clinicians and need a little review on excitatory amino acid neurotransmission; this is the 60-second excitatory neurotransmission review. So this is my cartoon glutamate receptor synapse.

And glutamate, as you all know, is the primary excitatory neurotransmitter in the nervous system. It can act on a variety of different receptor subtypes. The NMDA receptor is mainly postsynaptically localized; it is composed of multiple subunits or heteromeric just like GABA_A receptors and GABA_B receptors, and there are a variety of different subunits that can compose it. NMDA receptors are unique because they gate both calcium and sodium, and this calcium gating is one of the reasons that NMDA is thought to be so important for both normal activities like LTP learning and memory, as well as been implicated in models of excitotoxicity. It has also got two additional unique features. One is, it has got a voltage-dependent magnesium blockade, which means that the membrane needs to be depolarized for the channel to open, because that magnesium blockade has to be released. It also needs glycine as a coagonist before the receptor can be active. AMPA receptors are also postsynaptic, and they gate primarily sodium, but in certain circumstances, they can also gate calcium and that circumstance happens early in development as I will show you, which is when they lack a GluR2 subunit. So they are, again, heteromeric receptors composed of multiple subunits and usually they contain a GluR2 subunit; when they do not, they can gate both calcium and sodium. And that may be important early in development as I will mention in a minute.

Kainate receptors are a little more ubiquitously localized; they are both presynaptic, postsynaptic, and can be located on glia, and are also multimeric receptors composed of multiple subunits. They gate pretty exclusively sodium. And the last group is the metabotropic glutamate receptors. And they, like GABA_B receptors, are G protein coupled. And they also can be localized ubiquitously both presynaptically, postsynaptically. And on glia and come in 8 different subtypes that are grouped into 3 different functional groups.

So what happens during development of this system? Well we have 2 general sets of changes – quantitative changes and qualitative changes, and both of them go in the same direction, which is to promote excitability during early development. So this is a graph that I blatantly borrowed from a paper by Frances Jensen and Russell Sanchez, which is a very nice review of developmental changes. And this just summarizes work from a variety of laboratories that I have mentioned underneath looking at the density both of excitatory synapses as well as excitatory receptor number during the course of postnatal development. And what you can see is that big postnatal hump there in the first 2 postnatal weeks, where there is really an increase in both the number of synaptic terminals as well as a number of receptors in both the NMDA and the AMPA receptors' excitatory amino acid groups. Again, really positioning the neonatal brain to be highly excitable. In addition, there are qualitative changes within the individual receptors themselves. So we are back to my cartoon synapse here.

First of all, getting away from transporters for just a second, or getting away from receptors for just a moment to talk about transporters. Glutamate is taken up from the synaptic cleft by different sets of glutamate transporters. And one of them, GLT1, is primarily glially localized and does the lion's share of the work of removing glutamate from the synaptic cleft. That actually has very low levels of expression early in development, again which can enhance glutamate levels in the synapse. What about the receptors? Well, NMDA receptors have unique subunit composition early in development and this unique subunit composition contributes to several differences in their function. One, they are more calcium permeable so they let more calcium in, in immature neurons. They also have less magnesium blockade so what that means functionally is you do not have to depolarize the membrane as much for the receptor to open and for calcium to flow through. And finally, the excitatory postsynaptic currents are longer in immature neurons, again all related to changes in subunit composition, but all really enhancing the excitatory nature of these receptors.

AMPA receptors have additional changes. They have a change from what is called their flip to flop ratio. Functionally, the importance of that is that makes them desensitize more slowly, again so their channel stays open longer and it can gate more sodium and calcium. And probably most importantly, there is less of the GluR2 subunit that I talked about. So there are many more calcium-sensitive AMPA receptors present early in development. Finally, metabotropic receptors have increased turnover of PI, which enhances their signaling as well. So overall, what I think, what I hope I have shown you is that there are both quantitative changes in the increase in the number of excitatory

receptors and synapses as well as an enhancement of the properties of those individual receptors, all of which promote excitability in the immature brain. This in combination with the really slow development of the inhibitory system, I think we can see really sets up a situation where animals are going to have increased seizure susceptibility and humans will also have increased seizure susceptibility early in development. So the other side of the coin is, why do we care? Do early life seizures actually in any way alter normal patterns of neurotransmitter receptor development that may ultimately impact on a variety of aspects of the later developments such as seizure susceptibility as well as potentially learning, memory, and behavior.

So to look at this question, in our laboratory we have used the lithium pilocarpine model of status epilepticus. And in this model, the day before status epilepticus is induced, we inject the animals with lithium and then they are injected with pilocarpine the following day, either on postnatal day 10, again to mimic the sort of neonatal period, or a postnatal day 20, which is sort of more older childhood, for lack of a more specific timing. And the pilocarpine produces prolonged seizure activity, so this is really a model of true status epilepticus. These animals then are allowed to grow up normally, and then in adulthood we have studied them using both video EEG recording to see whether there is evidence of development of spontaneous seizures. We have also looked at seizure threshold to induce seizures, hippocampal morphology. And what I am going to talk about today, the GABA_A receptor, the physiology and subunit expression. And again, the physiology and subunit expression was all done in collaboration with my colleague, Dr. Doug Coulter and Dr. Robert Hsu in his laboratory, who did the physiologic studies.

So what we found was, in fact, this episode of early life status epilepticus does produce profound changes in GABA_A receptor expression and function in the animals when they become adults. So both GABA_A receptor expression overall is increased, which is about a 2-fold increase. And there is also selective increase in that $\alpha 1$ subunit that I talked about before. Robert in Doug's laboratory found that these changes in subunit expression were associated with an increased augmentation by the type 1 benzodiazepine agonist, zolpidem, again suggesting that what we were seeing at mRNA level was being translated into functional protein in these neurons.

I want to point out one other thing before I go by, which is the other thing that we found in the study, which was surprising to me when we initially noted it was that not only is there a change in the animals that are subjected to pilocarpine-induced status epilepticus, but the simple process of handling these animals to inject them, which is what was done to the group there that is labeled lithium. That alone, repetitive handling, briefly at postnatal day 9 and postnatal day 10, was also enough to impact the GABAergic system on a long-term basis. Now you will notice that that change is actually in the opposite direction of the change that we saw after status epilepticus, suggesting that there is unique signaling that can happen with even minor perturbations to the GABAergic system early in development and that very different alterations may happen with more major insults to the brain such as status epilepticus.

Now this is very different than what Doug and I saw when we looked at mature animals, so our initial collaboration looked at the effects of status epilepticus on adult animals. And what we found in that study was that there was actually a decrease in $\alpha 1$ subunit mRNA expression, again exactly the opposite of what we see after status in the immature brain. And there were changes in other subunits such as $\alpha 4$ and δ that were not prominent or not seen at all after status in the immature brain and that the changes that we saw in subunit expression again correlated with expected changes in the physiology such as decreased augmentation by zolpidem that were opposite to what we were seeing after status epilepticus in immature animals. So overall, we found significant age-dependent changes in the GABAergic system, depending on when status epilepticus occurred. Animals having increased $\alpha 1$ in dentate after status epilepticus at postnatal day 10 in the neonatal period, and decreased $\alpha 1$ expression in the same neurons after status epilepticus in adulthood. Now another very interesting change between these two groups is the difference in what happens to them in terms of development of epilepsy. So after status epilepticus in the neonatal period at least in our model in our hands, we have never seen an animal go on to develop spontaneous seizures. They do have a lowered seizure threshold, a seizure induction, using kainic acid, but they do not develop spontaneous seizures after weeks and months of video EEG recording. On the other hand, the adult animals in our model, 100% go on to develop spontaneous seizures. And they do it quickly with a mean length to about 4 days. And they have hundreds of seizures, so a profound difference in the epilepsy phenotype.

We wondered, we see this differential GABA_A-receptor expression and this difference of seizure activity; could these two things be related? Could the difference in $\alpha 1$ be a contributor to why the adult animals get epilepsy and the immature animals do not after status epilepticus. So we went to that intermediate time point that I mentioned to you, postnatal day 20. And at this age there is actually a split with about two-thirds of the animals going on to develop epilepsy after an episode of status epilepticus and one-third of the animals do not become epileptic after status epilepticus, and we looked at the GABA_A receptor expression in this group. And what we found was that like the postnatal day 10 group, those animals that did not get epilepsy after the midpoint status epilepticus had very high $\alpha 1$ mRNA levels; and that was also confirmed at a protein level using both Western blotting and immunohistochemistry. On the other hand, the animals that developed epilepsy had essentially unchanged $\alpha 1$ levels; so they were not as low as they were in the adult animals but they were not elevated the way that they were in the nonepileptic animals. And interestingly, their seizures are much less severe so the latency to seizure onset after status at postnatal day 20 is about 45 days and they have very few seizures – 1 to 2 a week, a much milder epilepsy phenotype than in the adult animals with status epilepticus. So this has made us actively pursue the question of whether $\alpha 1$ increases may actually be protective in dentate after status epilepticus and may be one of the contributing reasons to why neonatal animals do not develop seizures, do not develop spontaneous seizures or epilepsy after status epilepticus. And we have been investigating this using gene transfer technology to try to elevate $\alpha 1$ mRNA levels in collaboration with John Wolfe at University of Pennsylvania and Shelly Rostick at Boston University.

So on the other side, does anything happen to the excitatory receptors? Well the answer is yes. In addition to the changes that I showed you, there are selective changes in subunit expression in the AMPA system, particularly GluR₂ which seems to be selectively reduced in animals following status epilepticus at postnatal day 10. Again, functionally this could be very important because GluR₂, the absence of GluR₂, produces AMPA receptors that are much more permeable to calcium signaling. Now we are not the first people to see changes in GluR₂ after early life seizure activity. And in fact, Frances Jensen and members of her laboratory have looked at GluR₂ expression following hypoxia-induced seizures at exactly this age, postnatal day 10, and Russell Sanchez in their laboratory and others found that there was a profound reduction in GluR₂ mRNA expression. In CA1 also to some degree, and CA3, and I always like to believe in dentate, too, when you look at it there I think dentate is a little lower as well. But after 48 hours after hypoxia-induced status epilepticus at postnatal day 10, they then looked 96 hours later and found that there was a commensurate decrease in protein for the GluR₂ and also an increase in AMPA-mediated divalent cation permeability, which is a fancy way of saying calcium permeability, which is what the cobalt is a marker of. So again, these receptors functionally seem to gate more calcium. So that is in Figure A there, since the pointer is hard to see: Figure A here is the control animal; Figure B here is the animal 96 hours after hypoxia-induced seizures. And you can see a profound increase in the amount of cobalt, again a marker for calcium that is taken up after AMPA stimulation of these neurons.

Now interestingly, that can be blocked by AMPA receptor blockers. Now why is that important to us as epileptologists? Well one of the currently available antiepileptic drugs we know does have activity-attenuating AMPA receptor currents, and that would be topiramate. And Frances and her colleagues including Su Khee Kho in her laboratory actually went on to look at the effects of topiramate in this model and found that it could block the seizures quite effectively. And when it blocked these seizures, it also prevented later enhanced seizure susceptibility that she sees in this model. Even more interestingly, when they used topiramate after the episode of hypoxia-induced seizures, they could also block that increased seizure susceptibility later on. Again, suggesting that blocking the AMPA receptor may be one of those unique targets that may be particularly protective after seizures in the immature brain. And I would like to tell you that all of the action in the developing brain is in receptors, but that would not be fair, because it is not in fact true. And seizures have been shown to affect a variety of critical cell signaling molecules in the brain. And I just wanted to point out one particular example of this, which is changes in the hyperpolarization activated or h-current.

So this is worked on in the model of Dr. Talle Baram and colleagues where they used hypothermia to induce seizures in postnatal day 12 animals. And again, this is a model of the very common occurrence that we see as pediatric neurologists of febrile seizures. And what Talle and her colleagues found in collaboration with Thé Von Sholtash was that these seizures produce persistent hippocampal hyperexcitability in these animals. And Talle's laboratory has gone on to show that the subset of these animals actually go on to develop spontaneous seizures. Now they found that this hyperexcitability was attributable to changes, at least in part attributable to changes, in activity of the h-current

in CA1 neurons, and most recently they have shown what is displayed here on the slide, which is coordinated changes in the HCN1 proteins, which compose the h-current. And you can see here that in animals exposed to hyperthermic seizures as well as animals exposed to kainic acid seizures at postnatal day 12, that they have a profound and long-lasting decrease in HCN1 expression here at an mRNA level and a coordinate increase in expression of the HCN2 mRNA. And that is collaborated by changes in protein in the hippocampus. So again suggesting that a variety of receptors and other critical cell signaling proteins may all be altered in a long-term and possibly permanent fashion following early life seizure activity.

So I hope that I have been able to convince you that developmental changes in both excitatory and inhibitory neurotransmitters may contribute to the increased seizure susceptibility that we are all overly familiar with in the immature brain and that in turn when seizures do occur in the immature brain, they can disrupt a variety of normal activity-dependent patterns of receptor and other cell signaling proteins. And that some of these changes may actually in a way be protective, while others may contribute to enhanced seizure susceptibility and possibly even development of epilepsy later in life. Finally, it is a hope of all of us that work in developmental epilepsy models that what we will be able to do once we have identified these molecular changes is identify specific new therapeutic targets that may be used in the prevention of epilepsy following early life insults.

Thank you. I would like to (just quickly before I stop) to thank the people who actually did all the work. So I would like to thank all of the members of my laboratory, in particular, the people that worked on the data that I presented today – Dr. Uganda Rau here did all of the animal modeling work as well as some of the physiologic studies, and Dr. Gorgenzang there in back did the majority of the molecular biology work that I presented. And again, I am in constant debt to my wonderful collaborators, Dr. Doug Coulter at Penn, Dr. Shelly Russeck of Boston University, and Dr. John Wolfe, also at Penn, and of course none of this work would be possible without the financial support of the NINDS as well as the financial and emotional support of the Epilepsy Branch, particularly Margaret Jacobs as well as additional support from the Epilepsy Foundation, AES, and the Child Neurology Foundation.

Thank you. (Applause)

(Dr. Jeffrey Noebels)

Thank you, Amy, for that very elegant synthesis. And I think the major take-home message of all this is that the molecular aftermath of a seizure depends on the age. And so this adds a dimension that cannot be ignored as we analyze these various animal models of seizures.

Our next speaker, as you can see, the trend in these talks has been to go from basic to more real life situations, and that is where we are turning to next. Darrell Lewis is Professor of Pediatric Neurology at Duke, and he has been studying for quite a few years

now the natural history of a lesion that most of you know about, mesial temporal sclerosis, and has been imaging a longitudinal cohort of patients and is going to help us best understand what this problem is really all about. Darrell.

(Dr. Darrell Lewis)

Thank you very much, Jeff. Can you all hear me back there? If you cannot, jump up and down, wave your hand, or something like that. Thanks, Jeff, for inviting me here and I want to thank all the organizers who worked so hard to put this thing together and get us all straight this morning.

I am going to change the tune a little bit here and go to the clinic and talk to you about the clinical aspects of some of these problems that my colleagues have been discussing the basic mechanisms of. I want to go back one slide if I can. Jeff assigned me a topic: losing neurons. And rather than talk about what might be affecting my brain I thought I would talk about kids' brains and chose a phenomenon called mesial temporal sclerosis, which you are probably all familiar with, and the topic of selective vulnerability and mesial temporal sclerosis. Next slide please.

I would like to talk to you about some background information first – what is the relationship between mesial temporal sclerosis, which I will refer to from now on as MTS if you will allow me, and temporal lobe epilepsy just to get you in the setting. Then I am going to introduce this concept of multiple factors and the causation of MTS and then visit three hypotheses regarding increased vulnerability of the developing brain to injury and subsequent MTS and of course temporal lobe epilepsy. The three things we will touch on are dysgenesis, prior seizures, and genetic predisposition. MTS is particularly interesting as a phenomenon, because it has been known for over 100 years that this occurs and is associated with temporal lobe epilepsy, but in spite of all the wonderful advances you have heard about today, we still do not know how it develops in the human brain. It is still rather much of a mystery.

This is the MRI appearance of MTS. This is what we see in the clinic when we get a new patient in with intractable temporal lobe epilepsy and we do a T2-weighted coronal MRI of the brain. Here are the temporal lobes on the left and on the right, and this is the sclerotic hippocampus. There are three characteristics to the MRI appearance of MTS: atrophy, which reflects the loss of neurons in that hippocampus which is often profound; increased T2 signal intensity, which reflects the increase in gliosis in that hippocampus as a result of some prior event; and finally loss of internal architecture. If you look at this normal left hippocampus here, you can see some circumferential light and dark markings which reflect the layering of the pyramidal cell, and over here those are lost. This is what we see; this is the scene of the crime, but we do not know exactly what the crime was.

How about temporal lobe epilepsy in MTS? Well there are three things to think about – pathological examination reveals MTS in about 60% to 70% of temporal lobe epileptic cases coming to surgery, and the pathological material is seen to be the most common lesion in this very common form of intractable epilepsy in young adults, children, and

older adults as well. Removal of the sclerotic mesial temporal structures eliminates the seizures in about 70% to 80% of cases. So we think that somehow MTS is necessary for the clinical expression and generation of the seizures. Finally, subjects with temporal lobe epilepsy and MTS have histories of initial precipitating injuries, a term coined by Gary Mathern, and it is thought that these initial precipitating injuries may have something to do with the causation of the MTS.

Looking at the distribution of initial precipitating injuries in temporal lobe epilepsy, we see right off in this series by French, et al. of around 60 patients who went to surgery: The febrile seizures comprised the largest fraction, and this is consistent throughout all the studies that are available; in this study, 53%. It is never clear 30 or 40 years later what type of febrile seizure we are talking about, obviously. I am not talking about simple febrile seizures; we all know that they are not particularly connected with this problem, but perhaps longer ones and focal ones are. Other types of IPIs or initial precipitating injuries are CNS infection, head trauma, birth trauma, a mixture of things, and then there is always a group that has nothing in their history whatsoever to suggest why they should have this lesion, and we will touch on that.

So is this the equation we should be using? Is there an initial precipitating injury of some sort and that, combined with some particular individual vulnerability in the child affected, leads to MTS? Whereas the bulk of children who have an initial precipitating injury such as a long febrile seizure never go to MTS. They never have temporal lobe seizures. You know that from epidemiological studies that Dr. Shinnar and others have done so elegantly.

This is a variant of the two hit hypothesis that there has to be more than one injury sequentially to develop MTS. We will explore the vulnerability factors, at least some of them that are talked about in the literature today. This is an MRI of a child who we studied back in 1995. What made this particular child's brain vulnerable to a prolonged febrile seizure in MTS? We still do not know. She was two and four-twelfths-years old; she had 3 ½ hours of on and off very quiet partial seizures in the setting of the febrile illness; not convulsions. Twenty-four hours later you can see the swelling and increased signal in the right hippocampus and that suggests an acute injury to me, perhaps some sort of edema, maybe intracellular edema. Nine months later another MRI shows a slight decrease in size of that hippocampus, documented by volumetric studies and a persistent slight increase in T2 signal. This generates a whole bunch of questions in my mind. First of all, was that brain normal prior to the status epilepticus or was there some subtle abnormality, structural or molecular, that made this child vulnerable to these events? We will talk about dysgenesis as a possibility there. Is the result now an epileptogenic hippocampus? Is that hippocampus capable of generating independent seizure activity without fever or with presence of other lesser stressors? We will explore that hypothesis. Thirdly, was this child genetically prone to this outcome? In other words, genetically prone to a long febrile seizure and to the resulting neuronal injury in MTS and we will talk about that.

The first hypothesis then – dysgenesis predisposes to febrile seizures, neuronal injury and MTS. This is a very popular hypothesis in the literature, and there are numerous papers addressing aspects of this hypothesis. There is pre-existing dysgenesis, perhaps congenital, perhaps genetically determined, and I already talked to you about a lot of things that can happen in development that can go wrong and produce that sort of a problem. And the idea is that this pre-existing dysgenesis lowers the seizure threshold in the brain and with fever or some other stress we get seizures; not only does it lower the seizure threshold, but it also makes seizures more effective in causing neuronal injury and neuronal death – that is the idea. And hence, we get MTS. So we are exploring this hypothesis now.

Types of dysgenesis: Well, there is gross dysgenesis which is very obvious on MRI scans even with our current level of technology and I am talking about the advanced stuff you saw this morning. That is focal cortical dysgenesis; it very often involves a full thickness of the cortex and is obvious on an MRI. Then there is this diffuse severe stuff like lissencephaly, etc. We are not talking about that. That is not common in temporal lobe epilepsy; however, there is a more subtle form of dysgenesis. Some people refer to it as microdysgenesis; scattered, tiny microscopically diagnosed areas of varying types of abnormalities with subtle or no MRI findings. Usually no MRI findings. And this is more and more being described as common in temporal lobe epilepsy with MTS. Then there is a relatively new kid on the block – hippocampal dysgenesis in which one or both hippocampi are abnormally formed, and we will be talking about these two things right here. I am not trying to resurrect the theory of microdysgenesis and generalized epilepsy; I am not talking about that. I am talking about temporal lobe epilepsy. And so we will go from there.

Some features of microdysgenesis, just to give you an orientation of how difficult it might be to sort this stuff out microscopically. This is called a glioneuronal hamartia – those are mature neurons; and these are oligodendroglial-like cells with clear cytoplasm dark nuclei; significance unknown, but long ago and continuing into this day described in temporal lobe epilepsy as increased. And then we have the perivascular oligodendroglial cell clusters; again mysterious, but they are there and they are found in repeated studies in the white matter of the temporal lobe. And we have clustering of neuronal groups in the cortex lined up here too close together, sometimes with abnormal morphology in the neurons. And then we have a very common finding of increased numbers of neurons in the temporal lobe white matter; perhaps this goes along with Arnie's discussion of migration, some problem there.

Well, many of the investigators, and I am indebted to all of them for doing some very interesting work in this area, find increases in various types of microdysgenesis, but what I was looking for was papers with controls. I wanted to see if the investigators included tissue from control nonepileptic patients as well as from epileptic patients, and Professor Kasper kindly discussed his work with me and he looked at 29 controls in 47 temporal lobe cases and looked at 14 separate features of microdysgenesis; 14 separate things he

was looking for, and he only found that 4 of them were significantly increased in temporal lobe epilepsy versus controls. And those were the 4 I chose to illustrate for you – white matter neurons, hamartias, glial clusters, and neuron clusters. You can see that maybe 20% to 30% of the specimens have those phenomena but when you add it altogether, 30 of his 47 cases had 1 or more feature, so that is pretty common. So if microdysgenesis is playing a role in febrile seizure susceptibility in MTS, then one might expect to see an increased incidence of it in those individuals who have a history of febrile seizures and have MTS. The literature does not address this very often.

One study by Porter in 2003 looked at young temporal lobe epileptics, children – older children; 33 of them were taken to surgery, and they examined the tissue for microdysgenesis; 15 of them had a history of febrile convulsions and 18 did not. And the only thing you can see was this tendency for the febrile convulsion group to have more in the way of dysgenesis. It was not statistically significant. Professor Kasper has found the same thing in a similar study so we do not see evidence right now, the connection there.

The next thing we have to ask is could some of these so-called microdysgenesis lesions actually be normal? Could increased cell packing density in the temporal lobe neocortex give the appearance of clustering of neurons reported in temporal lobe epilepsy? We all know that in temporal lobe epilepsy there is often temporal lobe atrophy on the same side as the MTS and Bothwell et al. did some very careful stereological studies and found that this atrophy was accompanied by increased packing density and increased size of the neurons in the cortex and increased size of the neurons in the white matter as well, but no change in total cell number in temporal neocortex. So I am raising the question of whether or not that packing density could give rise to what looks like abnormal congenital dysgenetic clustering if you will.

Another question – are glioneuronal hamartias really abnormal? They have been described for decades in temporal lobe epilepsy, but Yachnis et al. in 2000, a very careful study, found that these could be found in the region of the amygdala and temporal horn of the lateral ventricle in the temporal lobe in a high percentage of controls. So we need to rethink some of this stuff perhaps.

Now I am going to switch gears again and talk about a different type of dysgenesis – familial hippocampal dysgenesis, proposed as a cause of febrile seizures and possibly MTS. Many people have described hippocampal dysgenesis prior to this article, and I chose this as the example. Fernandez in 1998 described 2 families; in each family there were multiple members with simple febrile seizures and abnormal left hippocampi. In each family there was one member who had gone so far as to actually develop MTS and temporal lobe epilepsy. And in Family A, that particular person had more than 50 simple febrile convulsions and in Family B, that individual who developed MTS had about 25 simple febrile convulsions. So what do they look like, these hippocampi? Well in Family B, basically it was a change in size of the head, not really much morphology. But in Family A, you can see that the hippocampus had lost its internal architecture and was somewhat flattened and also mesially shifted. So it was a recognizable MRI syndrome,

and it was associated with a clinical phenotype of febrile seizures. Interestingly, they were not focal febrile seizures as far as we could tell; they were not coming from their left side of the brain. So it is very difficult to understand what is going on here.

Now what do we have in the animal literature on dysgenesis and febrile seizures? Not much yet. Germano et al. used a model in which MAM was given to pregnant rats at day 15 to produce extensive, cortical and hippocampal dysgenesis. You will see the normal cortex shows nice layering and columnar organization; after MAM treatment, the rat has a brain that looks like spaghetti. In the hippocampus, the pyramidal cells are nicely lined in the normal baby rat, but if the baby rat got MAM during gestation, there are areas of disruption of the pyramidal cell layer. So this is a very different thing than we are talking about in humans; this is an extensive, profound, generalized dysgenesis. Now what these investigators found was that this dysgenesis increases neuronal injury due to hyperthermia. If the rat pups at 14 days of age were exposed to about 42 degree core temperature for about a minute or two, both the controls in the MAM pups would have seizures documented by EEG. Those are electrographic seizures, not clinical ones. But the MAM pups had more behavioral seizures. The seizures probably spread further in the brain, it produced behavioral limbic seizures. In addition, the MAM pups had more cell injury after hyperthermia, which was independent, interestingly enough, of the seizure activity. Whether they had seizures or not, they had more cell injury, and it did not seem to be a seizure-dependent phenomena. If you look here, after hyperthermia in the control mice, cresyl violet stained cells look pretty good, but in the MAM pup after hypothermia, and this one did not happen to have the clinical seizures, unless there is more loss of neurons and later on the cell counts found a decrease in neuron number.

So what can we say from all this study of dysgenesis and febrile seizures and MTS? First of all, I do not think we know at this point in time whether or not microdysgenesis increases the vulnerability to prolonged febrile seizures or to MTS in humans. I think it is very likely it might, but we do not know. We need more studies. Human hippocampal dysgenesis, on the other hand, limited to the hippocampus, may play a role in both febrile seizures and MTS in some small number of cases, but I think it is certainly not a large fraction of temporal lobe epilepsy. In animal models, severe dysgenesis can lower the seizure threshold and enhance hyperthermic injury, but we still do not know exactly how the seizures factor into all of this. So I would suggest that we need more studies in which the observers are blinded to what is the pathological tissue and what is the control tissue, and is there control tissue interspersed randomly with the pathological tissue. And we need to use that to clarify the role of dysgenesis and the causation of mesial temporal sclerosis.

The second hypothesis – I am going out on a limb here, this is a little bit dicey. Prolonged febrile seizures increase hippocampal excitability, enabling the gradual development of temporal lobe epilepsy and MTS. It is the second part that I threw in there. Let us see what you think of it. What I am suggesting is that we can have a normal child, a normal hippocampus, and for some reason, maybe genetic predisposition to febrile seizures or infection of some sort, get a prolonged febrile seizure and that can lower the seizure threshold. As Amy mentioned, Baram has been working on this. And

this can produce an epileptogenic hippocampus. The question is, can we go from there to here? Can we go from there to temporal lobe epilepsy and MTS?

First, I will start off this hypothesis with a discussion of the animal data. Dr. Baram and her colleagues have been working on the immature rat model of prolonged febrile seizures for quite some time. This is an age appropriate model in which PN day 10 rats, which is roughly equivalent to a 1 to 2 year old child, the age which we all have our febrile seizures. They heat these rats up with a hair dryer in a little beaker here with hot air, and they get about a 41 to 42 degree temperature, and 98% of them have a seizure. The EEG shows that this is a limbic seizure; it is 20 minutes in duration almost down to the minute in most of them; and there is no mortality and no morbidity. So it is a lot different than the seizure I showed you in that child of ours that had 2 ½ or was it 3 ½ hours of seizure activity; it is much briefer. And here you can see the cortical EEG with some movement artifact, the amygdala EEG with movement artifact. And you can see how when this clinical seizure starts, the amygdala starts firing here and the cortex remains relatively quiet. These prolonged febrile seizures, 20 minutes if you will accept that as prolonged, produce transient injury but no loss of cells. Here is the silver stain 24 hours later of the hippocampus, area CA1, you can see the dark neurons taking up silver, suggesting some sort of an injury. However, cell counts at 3 months, no difference in interneurons or in pyramidal cells in CA1 between the controls and those with seizures. So it is not generating mesial temporal sclerosis, but these hypothermic seizures do lower the hippocampal seizure threshold in what seems to be a permanent fashion. Because in the adult rats, if you take an adult rat who had a hypothermic seizure as an infant, you look at the baseline hippocampus electrical activity, it is normal. But if you give a subthreshold dose of kainic acid which normally would not produce a seizure, this rat has clinical and electrographic seizures to beat the band. Hyperthermia only if the rat was protected by benzodiazepines during the hyperthermia, no such problem in likewise controls. No such problem.

Now recently in Dr. Baram's lab has done some MRIs on rats. And this I find interesting, because I like to deal with MRIs. Hyperthermic seizures in these rats induce transient increased T2 signal in limbic areas, multiple limbic areas. Here you can see the piriform cortex, you can see the amygdala; about a week later you can see the hippocampus transiently lights up here. These changes are reversible and I do not think that we have any volumetric data yet to see whether there is any growth problem there. But it is very interesting in that there is a parallel in the human arena. Scott et al. have done some very nice work looking at febrile seizures in human infants and found that they also produced transient MRI changes in the hippocampus; again this is not the type of thing that we published in 1998 with these horrendously long seizures. These are seizures lasting only about 45 minutes on the average. MRIs were done in 21 infants within 5 days of febrile status; 30-90 minutes averaging 45 minutes duration. You look at them visually, you do not see anything; not much at all, only 5 had very subtle, questionable volume changes. If you measure the volume in the whole group, you find that the mean volume is increased compared to controls and if you measure the T2 relaxation time, which is measuring T2 intensity, you find that that is also increased if your MRI was done within 2 days of the event. But these things reverse; they go back to

normal; follow up 4 to 8 months later, 14 of these subjects showed that hippocampal volumes of T2 were no longer increased, but 2 of them, when they measured the ratio of right to left volumes and they compared that with limits of ratio in controls, these kids had an abnormal ratio. So did one hippocampus shrink? Do not know.

Another question is, are some of these children now left with an epileptogenic hippocampus like those baby rats? So we will take a leap of faith here and throw out a hypothesis; you can do with it what you want. Could enhanced hippocampal excitability lead to temporal lobe epilepsy and MTS? We know we get an increased excitability in rats; we may want to do it and get it in humans. Do prolonged febrile seizures in human infants produce lasting hippocampal hyperexcitability? Could stress then evoke subclinical limbic seizures in these hyperexcitable hippocampi? If you have ever worked up temporal lobe epilepsy patients for surgery and put electrodes in the hippocampus, you realize that they can have hippocampal seizures day in and day out while they are doing just fine, functioning perfectly well. So these things are all subclinical. Could repetitive hippocampal seizures such as that produce structural changes gradually progressing to MTS? Do not know.

The last hypothesis: genetic predisposition is a major factor in the development of MTS. And the example I will choose is familial mesial temporal lobe epilepsy with MTS. Fernando Sendez and his colleagues and collaborators have been studying this phenomenon, and they have come up with some very surprising data. They went to their clinics and they looked for families that met their criteria in their clinics of so-called familial mesial temporal lobe epilepsy. Those were families with two or more members who had this syndrome, mesial temporal lobe epilepsy. What were the criteria? Well clinical criteria were simple or complex partial seizures with an aura of rising epigastric sensation, fear, experiential stuff like déjà vu, jamais vu, and autonomic phenomena. Now this is compatible with mesial temporal lobe epilepsy and they could have no suggestion of any other partial epilepsy syndrome. Likewise, the EEG criteria were either classical temporal lobe spikes or nothing but they could not have anything else besides temporal lobe. Well I thought, gee, doing that you probably would not come up with very much, but, they found that mesial temporal sclerosis was common in these families and that correlated with epilepsy severity. Within any single family, you could have people who had terrible man-eating epilepsy or who had occasional seizures, or who had remitted. So there is a variation in phenotype.

They had 142 MRIs, subjects from 45 families, and they found hippocampal atrophy by volume measurements in 99 of those 142 MRIs, an increased T2 signal where it could be evaluated which was only in 97 of the MRIs was found in 67. So that is a lot of MTS. Atrophy was seen in about 90% of refractory cases versus around two thirds of benign cases, so it did correlate with the severity of the epilepsy as did the increased T2. However, I want you to think about this because atrophy and signal, the characteristics we associate with the MTS on the MRI, could be seen in some family members with benign mesial temporal lobe epilepsy who had remitted, or in some with no epilepsy at all. And likewise, there was no evidence for critical role febrile seizures in the genesis of MTS in these families; only 11% of subjects had seizures, and they were all simple

except for 1 or 2. There were trends for febrile seizures to be more frequent in refractory temporal lobe epilepsy and trends for it to be more frequent with a hippocampal atrophy, but those were only trends, not significant.

Fernando sent me this slide very kindly. It is a family of 8 siblings, 3 of which are pictured here with the MTS. You can see this 50-some-year-old individual with complex partial seizures since 4 years, only occasional seizures; has some nicely, visible right mesial temporal sclerosis. These are 2 fraternal twins, females, 47-year-old lady here; febrile seizure or two at 2 years of age and complex partial seizures since 9 years, and they are refractory. And you can see her severe MTS with a little bit of change on the left as well. Her sibling, her twin – no febrile seizures and only sporadic complex partial seizures also has MTS.

So I would like to throw out some stuff here, another few off-the-wall conclusions. First of all, there is no evidence febrile convulsions are essential for MTS in these families, and likewise, there is no evidence that other IPIs such as trauma or infection is playing a role. So I would suggest that apparently MTS can develop with minimal or no initial precipitating injury given the appropriate genotype, the power of the genes.

Well I have not been able to cover all of the wonderful literature out there and I am very grateful for the investigators who did help me with this. There are other theories that abound, but I am ready to buy in that MTS is multifactorial, that there is vulnerability plus injury, and that is where we get to MTS. The contribution of microdysgenesis, however, I am not ready to completely accept yet; I do not think we know how it plays a role here, and I think more work needs to be done. Hippocampal dysgenesis has been reported nicely in families and the evidence was fairly convincing; it may account for a small proportion of MTS, but not a whole lot of it; and we need confirmation of those types of familial forms. Genetic factors alone might cause MTS in the minority of cases, certainly not the bulk of our clinical patients, but they might increase vulnerability to the IPI in many, many more cases given the power they have in these families. But the specific genes of course remain to be identified.

Finally, a question – could prolonged febrile seizures, 40 minutes, 30 minutes, something like that, without MRI evidence of severe acute hippocampal injury, in other words a relatively normal looking MRI afterwards, cause a lasting hippocampal hyperexcitability and lead to MTS? Well how do we get the answers to those questions? Well we go to the NIH and we take advantage of their generosity, and we start a study, prospective study, to address some of these questions. It is called consequences of prolonged febrile seizures in childhood, the PI is Schlomo Shinnar. This is a multicenter study of infants presenting with febrile status epilepticus, and we will be following these infants until a lot of us have a lot less hair than we do now, if that is possible. But we plan to follow them for about 10 to 15 years; we will be doing multiple MRIs and we want to determine the incidence, outcome, and risk factors for MRI evidence of acute hippocampal injury during febrile status epilepticus, number one. We want to determine long-term consequences of febrile status epilepticus with and without MRI evidence of acute hippocampal injury. And there is that off-the-wall hypothesis. And finally, we want to

test for all candidate genes and predisposing to febrile status epilepticus and hippocampal injury. Good Luck.

Thank you very much. (Applause)

(Dr. Jeffrey Noebels)

Thank you, Darrell, for accepting this challenging problem and clarifying it a little somewhat for us. There are plenty of questions that remain, but I hope most of you feel the way I do that the kinds of basic studies that we heard before him will allow us sort of the ability to make sense once we get tissue and molecularly characterize it. And we may be able to start modeling certain cellular pathways that might lead to some of these issues and give rise to more concrete experiments that we can do.

Well, I would consider it an enormously lost opportunity if we did not use the Merritt-Putnam Symposium to make new friends for our society and our research, and so our next speaker I think is a preeminent example of that. On reading one of her articles, review articles, in the *New England Journal*, I realized that she has an enormous amount to teach us about neonatal brain injury. And it is really a pleasure to introduce Donna Ferriero, who is Professor of Pediatric Neurology and Director of the Neonatal Brain Disorder Center in San Francisco. And she is going to describe for us sort of the crescendo of what we have been trying to get to which is, what are our hopes and expectations and our ability to protect against some of these changes in the developing brain. And are there any insights from her studies of ischemic damage in the brain that we could ultimately employ in epilepsy as well. There are some overlapping pathologies and probably many differences as well; but, we are talking about some major new and exciting molecular targets for protection and hopefully someday repair of damaged neurons in immature brains.

So Donna Ferriero, I am happy that you are here.

(Dr. Donna Ferriero)

Thank you, Jeff, and thank you to the organizers of this wonderful symposium. It is always great to come and learn while you are being given an opportunity to speak and talk about some of the things that you have thought about. We are going to switch gears a little bit right now because we are going back to what I feel a little bit more comfortable with, and that is ischemia research. But I think you can apply this to any kind of insult to the developing brain, whether it is from seizure activity, trauma or hypoxia or ischemia, and I would like to tell you a story and help you, with me, rethink what we are doing in terms of trying to “protect neurons.” And we will see by the end of the talk that I am not quite sure we all know what we mean by protect and why we are focusing so much on just neurons.

So in the first slide, I would like to just propose to you some broad concepts. And the most important concept is that what we are talking about in regard to injury evolution is a process that occurs over a long period of time. And we have thought so much and so hard about interrupting and understanding cascades of injury that occur immediately at or near the time of the insult, when the seizure first occurs, when the ischemic insult first occurs. Looking closely at mechanisms of oxidative stress and excitotoxicity in an attempt to stop that prolonged cell death that occurs and we have learned through that that there are many intervening processes, like inflammation and multiple programmed cell death cascades, that impact on whether neurons and other cell types in the brain will survive.

So let us look at that little tiny yellow piece in this slide and think that maybe we might want to think a bit about repair and focus some of our efforts on processes that may occur days or perhaps weeks after an injury occurs. We know that, at least for ischemic brain injury, and I would suspect many of you know the data for this, for epileptic brain injury, that there is an evolution that occurs. And in this slide you can see in Panel A in a coronal section of a postnatal day 7 rat brain, that pale area of infarction that occurs 24 hours after the insult evolves to that B panel of a liquefied porencephalic cyst. That occurs in 7 days' time in the postnatal day rat brain. Does it occur in humans? Well McKinstry published this beautiful example of the same kind of injury. This is a MCA or middle cerebral artery occlusion stroke in a term newborn and, using multiple imaging modalities, you can see from this panel between the first day after that stroke occurred and 1 week later, there is an evolution of the injury that best can be seen, I think, in the large audience on that flair image where you see a very small area of injury getting bigger and bigger evolving. And we sit there in the nursery as neonatal neurologists and neonatologists and watch that injury evolve over time without knowing how to protect the neurons from the subsequent evolution of that process.

So it brings us to this idea of when then do we treat the injury; do we get in there and treat it immediately and then stop, which is most of the focus of injury research. We look at immediate therapy and then we look at outcomes of that immediate therapy. So let us think about targeting that response and thinking about what we mean by protection. So I would like to propose that what we are doing are a number of things when we study injury and protection. First, many of us have tried to salvage neurons. And we have done that with targeted pharmacotherapeutics, and there are hundreds and thousands of papers in both the epilepsy and in the trauma and in the ischemia fields showing that we can impact somewhat in that injury process if we target those therapies, and I am going to show you a little bit of that as the talk proceeds.

We can protect neurons, but this is not really feasible because what we mean by protection is really setting up a tolerant system, and that is preconditioning and I am going to talk about that briefly because it matters, especially for epileptic tolerance. And then I think we have to think about what we have heard all this morning about the beautiful developing brain, that there may be a possibility if we can better understand how neurons recreate themselves perhaps through adult cells that we might be able to repair the brain after it is damaged.

So then we have to say, we should not only think about protecting neurons because perhaps, like Arnie showed us earlier today, maybe some of those adult astrocytes if tweaked appropriately, might end up developing some new neurons for a damaged brain. So when we think about salvaging neurons, we can think about some of the postinsult therapy studies that have been done. And many investigators have thought about trying to change this cell death cascade by altering programmed cell death (apoptosis) both by some of the intrinsic and extrinsic mechanisms that govern those functions. This is too much to cover in this talk, but there are many good experimental models of this to show that we can impact at this level but not without a substantial cost, and I am going to show you what that cost might be. We can then focus on some of the intermediary pathways that occur after an injury occurs, and there has been much attention paid to the inflammatory response in regard to cell excitation in epilepsy and in ischemia, affecting both the cells that regulate that within the brain, the microglia, and the cell and the compounds, the cytokines, that effect that response, and I am going to give you an example of that.

If you use a drug that has a broad spectrum capability, an antibiotic that is shown to have anti-inflammatory effects in the nervous system in both chronic neurodegenerative diseases like MS, and in acute situations like ischemia and epilepsy, we can potentially protect cells. But let us look carefully at this. And if you look at this graph in A you are going to see the response at 24 hours; if you look at that last column, the light and dark gray bars, you see a small significant effect of the drug in preventing injury. But if you let those animals survive for a week and, as I told you that injury evolves over that period of time, the effect of minocycline given at or near the time of injury is no longer there. Now maybe if we give minocycline every single day, we might be able to modulate that inflammatory response, but studies are just under way now looking at prolonged infusion-type therapies to truly protect after an insult.

If we try to salvage the neurons by other mechanisms like changing metabolism with hypothermia, we can delay the process of evolution of injury. We can block receptor-mediated activity, and Amy showed you that beautiful cartoon of the glutamate synapse and the multiple postsynaptic receptors that can be modulated. And Frances Jensen and others have elegantly shown that these receptors can be modulated and inhibited to prevent cell death and subsequent seizure activity. We can also block the oxidative stress response with free radical scavengers; but free radicals are actually or reactive oxygen species, are also signalers of gene regulation that might be important for repair of the brain, and I am going to show you that in a minute.

Faye Silverstein recently published a beautiful paper using combinatorial therapy where she inhibited metabolism with hypothermia and then used AMPA receptor blockade. And when these modalities were used singly, there was no protection to this postnatal rat brain but when she looked at the brains with a combinatorial approach, she could get improvement and perhaps almost complete protection that was not only histologically shown in this slide here at postnatal day 35, but in that paper she also describes functional

preservation with this therapy, which is much more important than just focusing on the histology of what we see after an injury.

Getting back to looking at that modulation of those pharmacotherapeutic approaches, if we look at glutamate receptor blockade as an example, here was very nicely shown by Alnay's group a number of years ago, just the drug itself could cause apoptosis or enhance programmed cell death in the developing nervous system in the absence of injury. So this, we do not know if this is true for the human, but it does give us pause when we are using drugs like phenobarbital, benzodiazepines which this group has gone on to show also cause similar pictures to this one shown here in the postnatal day rat.

So salvaging neurons as I mentioned can also be done by scavengers like iron chelators that can block oxidative stress, but as I told you, reactive oxygen species are also controlling downstream gene regulation that may be involved in repair. So if we look at "radical scavenging" and I put this in quotes because this is not all that this drug does, desferoxamine, a drug used in humans to chelate iron for a number of disease processes. If we go back and recreate that middle cerebral artery occlusion in a post natal day 7 rat brain, we can show a modest improvement in volume preservation 7 days after the infarction is created, and that is shown here in this graph. However, the desferoxamine is doing a few other things and one of the other things it is doing is modulating an important gene that I would like to tell you about that is controlling a process, that is truly protective. And this process is called preconditioning. Preconditioning is actually a treatment with a sublethal stress that will actually provide protection when the full stress is given at a certain time period after, and I will show you a common paradigm, to prevent a severe brain insult.

This phenomenon is called tolerance, and it has been shown elegantly, at least in the adult rat after seizures. Before a hypoxic challenge is given, you can prevent the damage in the hippocampus by creating tolerance. Likewise, in neonatal rats, Jeff Gidday and his coworkers also gave a hypoxic challenge, brief, and then gave a full, severe hypoxic ischemic challenge 24 hours later and showed marked preservation or true protection of the brain.

So the paradigm looks like this – brief or prolonged stress, usually a 24-hour period so that certain downstream genes can be upregulated, and I am going to show you some of the important ones. The severe stress is then given, whether it is seizures or ischemia; and then gene and protein expression occurs; energy is preserved and that work was elegantly done by Susan Benucci and her coworkers; and true protection or no disturbance of the brain is seen in this situation. Obviously, this is not feasible, therapeutic paradigm, but it can give us an indication of how we might perturb the brain to condition itself at the time of stress.

And here, Marcelle Bergeron with us, showed that if you did this hypoxic preconditioning and then created hypoxia-ischemia in the developing rat, you could shift that injury paradigm to essentially no injury. And you can see in the hypoxia plus HI, a relatively preserved, beautifully preserved, brain at multiple levels through the striatum in

the first panel on the right and through the hippocampus in the second panel. And in that bar graph, you see those white bars showing the shift of histological injury towards normal with this preconditioning response. And when Marcelle Bergeron went back to look to see what was changing in the brain after this hypoxic preconditioning, she found the upregulation of a transcription factor called hypoxia-inducible factor 1. And hypoxia-inducible factor 1 has received much attention of late as the master regulator of homeostasis for oxygen in the brain, liver, and many organs in the body. And in the brain it does an enormous amount of wonderful things for us to think about if we want to truly protect the brain. And what HIF does as a transcription factor is create the ability of the brain to upregulate a whole host of target genes, over 50 target genes, that will allow the brain to repair itself or help itself at or near the time of injury. And hypoxia-inducible factor is actually a heterodimer that is made up of one subunit that is inducible with hypoxic stress and one that is constitutively expressed. It turns out that desferoxamine will inhibit enzymes that target this bound HIF to the proteasome for degradation. So desferoxamine is stabilizing this transcription factor which then binds with other elements to hypoxia-responsive elements on a number of genes. And here in this slide you can see those are genes that control the redox state of the cell, nitric oxide, growth factors like vascular endothelial growth factor and erythropoietin, which I would like to focus on for the rest of the talk, and a number of glucohomeostatic enzymes like glycolytic enzymes and the glucose transporters.

Marcelle Bergeron and coworkers went on to show that these target genes also came up, so it was not only the transcription factor, but the transcription factor was upregulating a number of these target genes during the hypoxic preconditioning period that may have set this stage for the brain to be protected after the hypoxic ischemic insult. Now if we are going to think about repair, maybe we can use some of this information that I just told you about preconditioning. Because with repair, we see the upregulation of a number of genes that are upregulated likewise during preconditioning. We see matrix remodeling, and we see massive upregulation of those growth factors, erythropoietin and vascular endothelial growth factor, I am now going to call it VEGF.

We also see new cells; we are not sure if these new cells remain, and I would like to show you some of that data. But first I would like to show you that if you create an ischemic insult in the brain, you can upregulate this target gene, excuse me – the transcription factor, hypoxia inducible factor, and its target genes, even without preconditioning the brain. So in a moderate ischemic insult like occluding the middle cerebral artery transiently, you can upregulate both hypoxia-inducible factor and its target gene very early after the injury. And when it is upregulated, it is upregulated in neurons, NeuN is a marker for neurons, and you can see if you do double immunofluorescence, staining in panel C when you merge those you see a number of the cells co-localizing VEGF in the new NeuN immunoreactive cells. Likewise, HIF, the transcription factor, is co-localized to the same cells that its target gene is co-localized to. Likewise, erythropoietin, the other growth factor, is upregulated after neonatal ischemia and can be sustained if we give desferoxamine therapy first as a scavenger, as we think, but possibly more to stabilize that hypoxia-inducible factor. And if you look at the last two bars under the Western blot, you will see that the desferoxamine animal has upregulated erythropoietin not only

early at 8 hours, but late at 1 week when we are hoping that repair is actually occurring. And what we can see when we look for erythropoietin is that it is co-localized to those NeuN neurons early at 8 hours, but at 1 week, the hormone is now in astrocytes, which we know are involved in repair processes throughout the developing nervous system and the mature nervous system.

So Jack Parent elegantly showed in a number of studies both in epileptic models and in ischemic models, that there is increased neurogenesis after the insult. And I just wanted to remind you of that data because he showed most importantly that the newly born cells did become region-specific neurons. In the neonatal brain, it is still a question mark. We know that cells are born; we know that they are populating damaged areas, but we are not quite sure whether they are sustainable or functional. And we are not quite sure what is happening with the subventricular zone, which is putting out most of those migrating neurons.

And here in a slide taken from Jack's paper on adult neurogenesis after a middle cerebral artery occlusion, this is a full panel of doublecortin immunoreactivity. Doublecortin stains migrating cells. In A and B you are seeing a cross section of a brain that underwent that right middle cerebral artery occlusion, the darker staining areas are doublecortin immunopositive cells that can be seen at higher power in C, D and E as clusters in D, and chains in E. You can see in G in the border of the lateral ventricle that most of these cells are coming from the subventricular zone in the most inferior portion of the SVZ. And if you look at that expanded SVZ on the infarcted side in I, and you see the box that is in I, you can look at that under higher power in J and see that that doublecortin amino reactivity is actually a beautiful chain of migrating neuroblasts that are heading toward that infarct in that striata.

In the immature brain, Jack Parent and Faye Silverstein collaborated to do a similar model; this time a hypoxia ischemia model in immature animals and looked early at about P12 7 days after, or 10 days after the insult, and then again a little bit later at P24. And in G, just to show you that some of those dividing cells by BrdU were NeuN positive. If you look at the box in G, it is reconstructed in H and you can see those cells that are reconstructed in a 3D format. But what you can see if you follow this down and I am just going to go quickly to J, that many of those BrdU-positive cells are actually GFAP positive which is an astrocytic marker and not a neuronal marker. So it looked like the sustainable cells, at least in this immature model, were actually glia at 2 weeks and not actually neurons in this model.

So we asked the question – could we repair after ischemia if we gave the brain a little help? And we chose to use the growth factor erythropoietin, because we knew it came up; we knew it came up in the right areas and we knew it was sustainable for a long period of time when we think repair is actually occurring. And indeed when we gave human erythropoietin to P7 rats after a transient middle cerebral artery occlusion, we were able to show that we could protect the brain with just one single dose after the insult. Erythropoietin, to remind people in this slide, is not in your syllabus but will be available on the web, has pleiotrophic effects, in no way are we claiming just the

morphogenic effect for what we see in our model, but we know that Epo and its receptor are present in the developing brain in very large quantities that diminish rapidly after birth. So it may give us a clue as to repair, and we know that it is induced by that transcription factor that I told you was so important in true protection or preconditioning the brain. So possibly, we can protect at least in neonatal ischemia models and possibly others. And there have been a whole host of papers that have just come out in the past 3 months showing protective effects in both in vivo and in vitro models of ischemia, and one paper that was recently published last month by Kumral and colleagues from Turkey, showing that functionality was preserved as well. And we also have shown that functionality is preserved at least 2 weeks after the injury when we look at some motor capabilities of our developing rats.

Now the real question is though – will this Epo exogenous therapy actually enhance or sustain some of those immature cells that are developing? And here I just give you a simple bar graph of doublecortin cell density to show you that in saline occluded SO animals on the infarcted side, we do see developing cells going to that infarct and we see more in the Epo-treated animals. This is very preliminary data, and what we would like to know is if those cells remain in sufficient numbers to actually create a functional benefit.

So in summary, I have given you a very brief overview of an opinion of protection in the developing nervous system that I think can be applied to all manners of insults that occur in the developing nervous system. True protection is not feasible, because we will not precondition unless we can consider minor seizures to be preconditioning stimuli that may create epileptic tolerance. I will leave that for you to fight over. Salvage may not be functional, because we may not be targeting the right area of the injury response, because we are not allowing for repair to occur; we are targeting too early and may be upsetting that tender balance between developmental programmed cell death and actual injurious cell death. Repair may be a good approach, but we have to think longer, and broader, and a bit outside our box in order to get some of these therapeutic studies done preclinically.

I think protecting neurons means protecting glia. So the concept of neuroprotection has to be maybe whited out, and we just have to think about protecting the brain rather than individual cell types within the brain. And although neurogenesis holds much potential for the future for repair of the damaged, especially neonatal, brain, I think functional neurogenesis has yet to be proven.

So I would like to thank you for staying and listening to the last comments of this wonderful symposium, and I would like to acknowledge all the support that I have received and in particular for this talk, as usual from NINDS and also from Ortho-McNeil-Johnson & Johnson for supplying the human erythropoietin and a number of people, collaborators, and big workers in the lab who have done all the work that I have shown you.

Thank you very much. (Applause)

(Dr. Jeff Noebels)

Thank you very much, Donna, for that important and provocative talk. I can assure you the fighting started already the minute you said the word preconditioning, and I also heard some people calling on their cell phones to order primers for HIF-1 and Epo. So we will start soon. Well a number of you have given us some great questions, so I am going to invite the other speakers to join us and for those of you who would like to stay for 10 more minutes, we'll answer these questions. Thank you very much.

Q: I am going to ask as many questions as I can and so we will look for short answers. Dr. Ellen Grant, is it possible to use DSI in a setting of an ictal event?

A: The DSI sequence takes about a minimum of 15 to 20 minutes to perform, so the ictal event would have to last at least that long. Motion in fact is always a problem, so we would want an ictal event that did not come with a lot of motor activity. But given those limitations, it is possible.

Q: Are there any known harmful effects of MRI repeatedly to the neonatal brain, especially the premature brain?

A: None that we know of.

Q: Can newer techniques of neuroimaging be applied to fetal MRI regarding the maternal placental fetal distress that contribute to the risk for epilepsy?

A: Actually, we are looking into that right now. The big advances are some of the faster imaging that comes with technology, so we are looking at getting spectra of the placenta, of the fetal brain, also trying to do more sophisticated diffusion imaging in the newborn as well, too.

Q: And finally, if it takes about a half hour to apply the data, how long does it take again for postprocessing information to get these spectacular pictures you showed?

A: It takes, the postprocessing is a couple of hours in these data sets. We are working on some newer technology, computer technology, to speed up the processing time. Also, the software that we interact with right now to display these images is quite laborious but we are also developing a software to allow a more user friendly interface.

Q: Thank you. Arnold Kriegstein, is it known how often each radial glial cell enters division cycles?

A: No, that is not entirely known. There is evidence that there are subsets of radial glia that may behave differently from other subsets. There is evidence from Rakic's lab some many years ago that radial glia both include those that are cycling over short periods of time and some that apparently cycle over much longer periods of time, so that really has not been thoroughly explored. I should mention that in the mouse it looks like there are

11 neurogenetic cell cycles from the beginning to the end of cortical neurogenesis, but it is unclear whether all the progenitors participate in each of those segments.

Q: What about Kahal retsial cells? How do they relate to the radial glial cells? Are they interneurons which originate from the ventral pallium? Are they GABAergic?

A: Yes. That is not a yes to those questions. It is very interesting about Kahal retsial cells. Fate mapping of cells that derive from radial glia have been done by two labs; one, Magdalena's lab in Germany and the other, Matt Hines and Goric Frechelle and colleagues. And they used a ROSA reporter mouse in a strategy that allowed them to see cells that had at some point been derived from radial glial cells. In at least one of those studies, the second one I mentioned, Kahal retsial cells were labeled. So they do appear to derive from radial glia. The second half of that question; they do not seem to come from the radial glia in the dorsal cortex that I talked about and Sam Pleasure in particular has evidence that he will present I think at a symposium and a workshop, investigators workshop tomorrow, that some of these Kahal retsial cells come from area in the medial cortex known as the cortical hem, but it is still being worked out.

Q: Pat Levitt, how do you know the parvalbumen cells are missing? Could they have just stopped making parvalbumen and still be present?

A: That is a good question, and it is an experimental conundrum, because we typically depend upon markers to identify subpopulations of cells. And we know now that expression of those markers is highly dependent upon activity in other factors. In the initial studies that we did in the uPAR knockout mouse, we actually saw a, what I would term as a generically, as a logjam down in the ventral telenceph level. We saw cells that were labeled that did not migrate out. But in other background strains in fact, we think that some of the cells do get up there but they just do not express to typical levels of PAR.

Q: Could you speculate on the harmful effects of environmental agents on the subpopulations of neurons as they are migrating? Certain signaling pathways?

A: That must be from a reporter who is looking for...some environmentalist! Okay – so there has not been an enormous amount of work done in the area of looking specifically at subpopulations of GABAergic cells, but we do know that modulating prenatally neurotransmitter activity, even transmitter activity such as monoamine, , serotonin, and dopamine can actually affect the differentiation of GABAergic cell populations. We also know that prenatal and perinatal stressors have a dramatic effect on the expression of subpopulations on subtypes of GABAergic receptors, which are permanent and can affect the inhibitory networks as they function in the cortex. So certainly environmental factors do have an effect.

Q: Amy, given that GABA is depolarizing and excitatory in immature brain, how do you explain the fact that benzodiazepines and barbiturates are effective against seizures in status epilepticus in neonates and pediatric patients?

A: Well I think we have clinical studies that suggest that although these agents are effective in some patients, there are many patients, particularly the preterm infant population, where we have very poor efficacy for these drugs. Mark Sherer and Mike Kainer have looked at that question in that population and really showed that only about 50% of neonates with seizures are effectively treated with at least Phenobarbital, which is what was included in that study. Now that still leaves 50% obviously that were effectively treated, and I think that speaks to the fact that you may to some degree be able to still get efficacy by pushing doses really high and if you think about what we do clinically, frequently we use dosages up at 50 to 60 per kilo to control these kids, which are not doses that we either use or whatever be tolerated in older patients. So it is never an all or none phenomenon. There are still GABA receptors there; it is a gradual transition from excitatory to inhibitory. So some of the receptors, even at term are undoubtedly inhibitory and there are some GABA receptors there. But as far as efficiency of a therapeutic target, I think it is much lower in neonates than it is in older patients.

Q: Here is a yes or no question. How should we be treating neonatal seizures if not with GABA agonists?

A: Okay, well I have disclosed that I will not discuss unapproved usages of antiepileptic drugs, but I will say that if you look at the targets that are available in the neonatal brain as we talked about, there is a preponderance of AMPA receptors. And I think that using AMPA receptor blockers or things that attenuate AMPA receptors, and there are some currently available medications on the market that do that such as topiramate may be a more efficacious way of doing it. There is also, there is a recent study by Nick Coolas and Dan Johnson that show that lamotrigine has some efficacy at HCN channels. And again, changes in HCN channels seem to be important in some animal models of early life seizures so again, that may be a medication that could be considered in this population.

Q: And would you use magnesium for seizures in neonates?

A: That actually is a very interesting question. I mean, NMDA receptor blockade is certainly something that people have thought of, and I saw a question that related to hyperglycinemia and the severe seizures in that population. The problem with NMDA receptor blockade is that truly efficient blockade also disrupts the critical, normal processes of the NMDA receptor during development, so it is hyperexcitability that is present in the immature brain is not there to create seizures. It is there to enhance learning and memory and all the normal processes that are exuberant in the developing brain. And we have to have a careful balancing act between not blocking those normal processes and treating seizures. So I know that hyperglycinemia for example, dextromethorphan has been utilized with some degree of success, although certainly not a great deal of success. But I think that totally shutting down NMDA receptor activity is potentially a problem.

Q: Darrell, could you discuss the asymmetry of hippocampal damage in MTS and comment on its significance, and are there control groups in this longitudinal study?

A: Well the asymmetry is exactly that. There is usually bilateral injury. And we have seen that in our children with prolonged focal febrile seizures, that both hippocampi show some effect on growth. And the pathological studies have shown the same thing. Now why is the brain asymmetric; why isn't hippocampal sclerosis asymmetric? I do not think we know, although one interesting hypothesis recently has been human herpes virus 6 and 7 and its role in febrile seizures and MTS, and we have published a paper not long ago on bone marrow transplant patients showing that recrudescence of that infection in the limbic system is often asymmetric. So perhaps sometime the inciting agent itself is asymmetric. And what was the other question, Jim?

Q: Well it was just wondering again about the functional significance of lateralization? I do not know if you can comment on that briefly.

A: Functional significance. Given that right equals left, I do not know if there is any functional significance whatsoever other than the fact that in temporal lobe epilepsy of course, right hippocampal injury is much less associated with verbal memory function problems than left.

Q: Finally, two questions for Donna. Should we consider Epo in premature infants with HIF? It says, given their predisposition to anemia they receive Epo anyway. Maybe they mean HIV?

A: In many nurseries in the country, Epo is still used for preterm infants and Nancy Newton and Bob Katz, who at UCSF looked at long-term outcome after Epo usage in that population, and found no significant adverse effect of the drug itself. They did not do a detailed analysis of cognition, behavior, and things like that, but we know at least in the very premature baby that Epo does appear to be safe in select populations.

Q: Okay. And finally, the comment is IUGR preterms, secondary to placental insufficiency should be neuroprotected. But they do have vascular accidents. Can you explain?

A: They should be protected, but they have stroke anyway.

A: (Donna) If I understand the question, you are saying that IUGR might be a preconditioning response to further ischemia? There are mixed data on this question actually, because it is true that some babies with interuterine growth retardation do seem to weather the neonatal period better, but recently a number of studies have shown, I think Yvonne Wu has just looked at this in neonatal stroke population in a large Kaiser cohort, that IUGR was actually one of the risk factors for neonatal stroke. So I think the data are not in, and it may, something as broad reaching and multisystem as IUGR, may not be what we are looking at in terms of a real preconditioning response, which is truly a

selective sublethal stimulus directed against the big stimulus, which is exactly the same 24 hours later.

(Jeff Noebels)

So it is an interesting subset to think about. Well I think this brings us to a close and I want to thank all of you, our audience, remaining, and our speakers for joining us. We had a great time and I hope you found it interesting. There are lunchboxes available for you to pick up on your way out and I was told you can take them to the La Nouvelle Ballroom for dining room tables to eat, and I guess that makes it nouvelle cuisine, if you will. Thank you very much. (Applause)