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**PROFESSOR:** The main topic today is the methods we use to study neuroanatomy, in particular connections in the brain. We didn't get through the discussion of slides from the pictures from chapter one of the book. And I know that interpreting presynaptic inhibition and presynaptic facilitation that happens in synapses with this arrangement, which you see on one of the pictures from last time.

So I'd like to go over that. And are there other questions from last time? Because I know if you have questions after a class, you don't get a chance to answer them, you can always send them to me by email, or you can just bring them up at the beginning of the next class. I really want to get your questions answered.

And as I mentioned only after the class to some students that were still up here, if you find terms that you don't think are adequately defined in the textbook, or you think should be defined in the glossary, please let me know, or let Caitlin know, because I will get them defined, and I will add them to the glossary. And that will affect everybody reading the book, because I'm going to post- I haven't posted it yet on the MIT press website-- but we have a provision for that.

And so when the book comes out, I want that glossary to be there. I just want to go over it a little bit more before I post it, but you already have it because it's on Stellar. And I'd be happy to answer other questions.

Most of those words come from last year's class. And I spent a lot of time writing definitions. And the words were given to me by the teaching assistant, so I'm sure they were words that she wanted defined. Maybe you students will find others that should be there. And there's probably a few defined there that weren't in the book, or maybe they were part of a figure or something, and she wanted a little more detail.

OK. So consider this kind of connection that you see right here. This is a usual kind of synapse. It's a bouton with only one synapse. Remember, if a bouton is big enough, it often makes several synapses with various processes on different sides of the bouton. But this is the way it's usually pictured.

This would be probably an excitatory synapse. They're usually fairly asymmetric. The presynaptic thickening here is varying in thickness. And then there's a postsynaptic thickening. And you always find this connection with vesicles on the presynaptic side. We know for sure now that they contain little packets of neurotransmitters. And if there are peptide modulators in the synapse in addition, they're generally in separate vesicles, but they could also be released and affect the action of the synapse.

But what about a synapse on the bouton that's making the synapse? What is its influence? So think about it now. Let's say that just before or during the time this action potential is arriving over this axon, the axon making this synapse on cell two here-- we'll call this cell one, this cell two-- this axon fires and reduces the membrane potential here.

You can't really call it an-- it's like an excitatory postsynaptic potential, but there's nothing too excited. It's not near an axon hillock. It can't affect directly the firing of an action potential here. What does it do, then?

Well, if it reduces the membrane potential here, when the action potential arrives here, it doesn't depolarize the membrane as much because the membrane potential was lower. So we call that presynaptic inhibition, even though it's a depolarization with that synapse, because it decreases the amount of neurotransmitter released here, because the neurotransmitter released depends on that change in membrane potential.

OK. So now let's say it causes hyperpolarization of the membrane, uses a different neurotransmitter, and different receptors here on the postsynaptic side. It causes a hyper-polarization. What will be the effect then?

Then, even more neurotransmitter will be released here. So we call it presynaptic facilitation. It increases the effectiveness of that synapse. That's the basic explanation for presynaptic inhibition and presynaptic facilitation. They depend on axoaxonal synapses.

It's common, for example, in synapses of the dorsal root axons on the secondary sensory cells in the dorsal horn of the spinal cord. And those effects were discovered just with large electrodes by physiologists who were extremely clever at using electrodes before we had microelectrodes.

All right. This is just an explanation for why all these interconnections in the CNS had to evolve. There had to be communication between different parts of the organization because the organism is big. So it's like the question of how does the right hand know what the left hand is doing? You've got to have interconnections.

How does the right hand know what the left hand is doing? Connections up here. And how do we study connections? Well, the methods of neuroanatomy. Sometimes we call it neuromorphology.

But there are other methods that can be used-- electrophysiological methods in particular. We'll mention one of them. It's been known since Sherrington's time. But also, there are chemical means. There are behavioral studies that play a role. And now, of course, various imaging methods.

But I just want you to pay attention to this remark here. The imaging methods are very limited for the study of pathways and connections. They do show you pathways now, and you'll see a picture of that. But they are not-- they don't give you certain knowledge of an actual connection.

So let's look at neuroanatomical methods. They start with fixing the tissue. That means both preserving the tissue and fixing it, because the tissue is very gelatinous. You need some way to make it firmer. And that's what fixatives do. The most common fixatives are the aldehydes-- formaldehyde, glutaraldehyde. Glutaraldehyde makes the tissue so hard the stains don't penetrate very well, but it's very useful for electron microscopy.

And then, of course, you cut the brain. It shows a little cartoon here that's, I think, in the book. You make a thin section. You mount the section on the slides. And in some stains, you do the stain before you mount them. But usually you're mounting them on slides-- often many sections per slide if it's a small brain. And then you dry them, go through a set procedure, and put them through the staining solutions in order to get the structures visible, the structures you need to see.

So what are some of the-- I think we talked about this last time. Can you just tell me what some of those techniques are if we want to do cytoarchitecture, we want to see the arrangement of cell in the brain? What do we do? Yes?

**AUDIENCE:** [INAUDIBLE].

**PROFESSOR:** Yes, that's a good one. We can get very nice pictures of fiber architecture that way. Yes?

**AUDIENCE:** [INAUDIBLE].

**PROFESSOR:** Nissl stains for cell bodies. That's the most common one. OK, and for fibers, if you want to do fiber architecture, I actually have a separate question here. What besides the silver stains do we stain for? What's the usual black-looking stain, where fibers are black or blue? Myelin-- they're myelin stains. Sure, that misses a lot of the small fibers, of course. But that's been a very important method.

And myelin stains and certain cell stains-- not the cresyl violet that we use in the laboratory but [INAUDIBLE] stains are used by pathologists in human tissue when they're evaluating the tissue. And so it's very common in the pathology literature to read, for example, hematoxylin and eosin stains, because eosin is a rapidly applied cell stain-- not as good as with cytoarchitecture, but useful for interpreting damaged areas in the brain. And iron hematoxylin stains the fibers. Yes?

**AUDIENCE:** What about tracers?

**PROFESSOR:** Tracers-- here we're only talking about cytoarchitecture and fiber architecture. You need tracers if you want to follow a specific axon pathway and find out where it goes. So that's the main topic today.

But here's an example of cytoarchitecture in an experimental animal. This is from a rat. And it's from one of Larry Swanson's beautiful atlases. And it's a section through the tween brain of a rat.

And you can see here that I've labeled a really obvious cell group pair in the hypothalamus. It's the ventromedial hypothalamic nucleus. And you can see some evidence that there are differences from one region to the other. Like right here, you can see the medial part of this area is very different from the lateral part. And that whole region is a little bit different from the area around it. That's cytoarchitecture.

Anatomists try to mark boundaries of these different cell groups if they can consistently see, often using more than one method to make them up. These are the cortical areas. Remember, I said the hemispheres sort of grow out of the diencephalon. You see that very clearly here. The connection's rather quick here, but there's a lot of axons in here.

And you say, well, actually there's a lot of cells there. Well, look at this. Right there, right here, right here, right here-- these are all axons. So what are the cells? What is the cell making the myelin? Oligodendrocytes, remember?

So you stain the Nissl substance in the oligodendrocytes. And for example, here the fiber is coming from the hippocampus. You see a lot of oligodendrocytes there too. The neurons are bigger. We're normally looking at it in lower power. But when you blow it up, you can easily see those differences.

And then look at the cortex. What's the most characteristic feature of these cortical layers? Here's neocortex up here. Here's olfactory cortex. Layers, lamination. And you can see very clear evidence of layers here, and we'll be talking a lot more about that.

**AUDIENCE:** [INAUDIBLE].

**PROFESSOR:** This is the habenula, yes.

**AUDIENCE:** Why is it dark?

**PROFESSOR:** The medial habenular nucleus is much more densely packed and stains more darkly. There's more Nissl substance.

This is a more magnified view to show you another example of cytoarchitecture. This is a frequently used example, because the boundary is so clear. The boundaries aren't always this clear.

But look at the difference between the cortex on the right and the cortex on the left. It's area 17 on the right here and area 18 on the left-- really clear. Area four here goes all the way from here down to-- it's right here, actually. This is because of the sub-layers in layer four. OK, I've shown it over here.

And you can see the differentiation of the different layers. And you see that some of the layers have sublayers. Layer six, even in the rat and mouse, very clear sub-layer, same as in human. Layer one, by the way, has very few neurons. We call it a zonal layer. It does have a few neurons, but very few.

What is there? [AUDIO OUT] neurons. It's the same thickness as other layers. What's in all that tissue? What's in the neuropil between the cell bodies? Dendrites and axons, and of course glial cells. But it's mostly dendrites and axons.

The pyramidal cells down here send their dendrite, many of them. The pyramidal cell has an apical dendrite, goes right up and arborizes at the surface. And we'll see pictures of that. But even these cells down here, a pyramidal cell will send its dendrite up like this and branch.

Well, what's it doing that for? It has other branches down here and down here. What's it doing that for? Because they're axons. They're terminating there. Axons from other cells in the cortex and axons from the thalamus.

Here's an example of fiber architecture. Here the myelin stain is not super-sensitive. It is missing the smaller myelinated axons. This is a human brain, but you do see the big bundles of myelinated axons very clearly. What is that huge bundle at the base? Cerebral peduncle coming from neocortex. And I've labeled it there for you.

And here's another myelin stain, but this one is a super-sensitive one. Because here, I've actually prepared this in my lab. This was done on a developing hamster. We were using at that time a newly discovered molecule discovered in oligodendrocyte membranes. And an antibody to it was developed. And when that became available, we applied it to developing animals.

And we could see, even before the myelin stains could stain the myelin, we would see these oligodendrocyte membranes. This is just a blow-up where you can actually see single cells there. So it gives you a very sensitive picture of the myelin. So you can see here, you don't see any-- this is the area we're dealing with in the human. There, you'll see there's actually a lot of myelin there. It's just a little harder to stain than in those larger axons in the peduncle.

Now, here's another use of the myelin stain. This is from a developing human, seven-week human. It's a horizontal section. So this is caudal back here, where you see the cerebellum. And there's the hemisphere. And there's the thalamus and some midbrain in between.

So what is interesting here? Well, in the hemisphere, you'll note there's a lot of myelin in the brain stem-- thalamus, midbrain, and hindbrain. Cerebellum is part of the hindbrain. But in the cortex, just a few fiber pathways are starting to myelinate. The earliest ones to myelinate are going to-- somatosensory areas, the primary auditory areas, the primary visual areas. They're very early to myelinate.

And that's from the work of Paul Flechsig. He was very well known for these studies of myelination, or myelinization. They're words that mean the same thing. Myelination, or myelinization. The development of myelin.

Now, if we wait a lot longer, we can't discriminate these three pathways from all the others. There would be so much myelin. Any large brain where axons are long, there's a lot of myelin on the axons. It's the only way the conduction can be fast enough to get efficient information processing. This is the same as one I have in the book, except I think I took out all these German labels.

OK. Now, what other methods do we have? You can use immunohistochemistry for your anatomical studies. What do we mean by immunohistochemistry? First of all, just say histochemical methods. Any method for specifically staining a specific chemical in the brain, in the tissue.

If it's immunohistochemistry, you use an antibody to bind to a specific chemical or a specific molecule type. And this just shows an example of immunohistochemistry. This is a section stained by a former MIT graduate student, Miles Herkenham, who's worked at the NIH laboratories for a long time. And he's converted the intensity of staining two colors here, just to make it more dramatic. He's binding to opioid receptors with his antibody and showing that the opioid receptors are much denser in some areas than others.

And then I want to ask a question here. How was histochemistry used for comparing forebrain structures in mammals and birds? It was really important study because it raised a big issue. This is the picture-- sorry, this one.

Here's a mammal. And note that the cortical areas there don't have a lot of acetylcholine esterase stained with the histochemical method for that molecule. Whereas the subcortical regions, that's all corpus striatal region, the corpus striatum, has a lot of acetylcholine esterase.

So then, you look at the pigeon and you see these areas down here have all the acetylcholine esterase. But there's this really large area up above that doesn't appear to be cortex. They have a little cortex visible here, and there's a little bit of cortex out here. You can't see the lamination very well.

But what is all that? You see, it raised this big question about comparative anatomy. We were thinking before then that birds and reptiles, they have this huge corpus striatum. This raised the question that maybe it's not really corpus striatum. Maybe this is the only real corpus striatum in the pigeon. That turned out to be true.

This is just another example in the mammal of the same stain, staining for acetylcholine esterase. This is from Ann Graybiel's work, where she showed this patchiness of acetylcholine axons in the deeper, just below the optic fiber layer coming into the superior colliculus. And a lot there in the superficial gray.

So histochemistry is more specific than the general cell and myelin and other axon stains that we're talking about, much more specific. And of course, there may be other molecules that you can stain for besides acetylcholine esterase. But that's been a common one in neuroanatomical studies.

And then we have newer technology. Now we can look for gene expression patterns in the CNS. And you can see when I did this, I called it recent. That was 2007. I point out here you can find online expression patterns of about 200,000 genes. I don't know if that's actually true, but that's what I have been told. And here's a Nissl stain of a little piece of cortex in the upper left.

And here's one that's showing cells expressing a particular molecule, but they're only in layer two. Here's one that's in layer two and three. Here's one that's only in the deeper part of layer three. Here's one in layer four. Here's one in layer five. Here's one in layer six, all the parts of layer six. And here's only layer six B.

So you see how specific they can be. And I've just given a reference where that was published. And it was a huge amount of work, getting all this to work. There was 108 authors of that paper.

Now, I don't think we talked about this-- the advantages and disadvantages of using the Golgi method for tracing interconnections of structures in the central nervous system. We know the Golgi method has been available for a long time. It was discovered before-- it was the end of the 19th century.

It was developed by Camillo Golgi but put to great use by the Spanish neuroanatomist Ramon y Cajal. What were the advantages and disadvantages? Well, you also should know who Ramon y Cajal was. In many ways, he's the father of modern neuroanatomy, but there were plenty of neuroanatomists already doing pretty interesting work.

But he put this one method to incredible use because of the advantages of the Golgi method. This is just one picture of a brain stain with the Golgi Cox method. It does not get every cell. It seems to randomly stain certain cells and not others. I played with that a little bit. I found out that just jostling the tissue a little bit when it was lightly fixed or hardly fixed at all altered the staining patterns.

I could sometimes get many more cells, get so many cells stained that it wasn't useful. Because the most useful part of Golgi is just that it doesn't stain everything. Because when it does stain, it tends to fill the whole cell-- dendrites, and in many cases even the axon.

And here in the background, a Nissl stain has been used, with only partial success, to show all the other cells. And that's what you see here too, but this is a more magnified. It shows some stellate neurons in the cortex surrounded by many other neurons that are not stained.

And here's a picture of Ramon y Cajal himself. See, it's a very simple microscope, a monocular microscope. Now we usually use binocular scopes. But you notice, he appears to be doodling. He's not even looking here. What is he doing? Is he doodling?

No. He studied through the microscope, memorized what he was looking at, then he did his drawing, completely from memory. He had incredible visual memory. And now people generally use tracing methods because the optics is more advanced. And we're finding that Cajal was almost always correct. So we know his memory was accurate.

OK, these are just some examples of his beautiful pictures. This is a section of the spinal cord, and he's showing axons there, coming-- these are dorsal root axons, terminating in the upper layers of the dorsal horn and then in the lower part of the dorsal horn.

And here you see-- we call these 1A axons. They're coming from muscle fibers, carrying sensory input from muscle fibers, and terminating right in the ventral horn, some of them directly on motor neurons. And here, on the other side, he shows some terminating in the medial part of the gray matter of the [INAUDIBLE]. That's in an area that is carrying input on proprioception, and the cells there send their axons up to the cerebellum.

This is an interesting one. He's just showing the ventral part of the spinal cord. And here, he's done something a little different. He's drawn all the dendrites and the cell bodies in black. Notice some of the dendrites go right across midline. And he's drawn the axons in red. So when you see an axon going out of the cord, you know that's from a motor neuron. That's the definition of a motor neuron. It sends its axon out.

He was very careful about figuring out the differences in detail of axons and dendrites seen with the Golgi method. And you can see, with this method, he could see connections. The big disadvantage of the Golgi method is it's almost impossible to follow the axon for very big distances. So if you're trying to find the connection, say, of brain cells with spinal cord cells, you'll never be able to follow it all the way with Golgi. You can do it with painstaking reconstructions, but we don't have a month to study one connection, so very few people have done it.

So I like to point that he was the first anatomist to see something that people had postulated-- and Sherrington knew, had physiological data for-- that some connections went directly from the sensory side to the motor side. Reflex-type connections, sometimes with even a single synapse, the monosynaptic reflex.

Cajal, he draws them here. He thought those were coming from the skin. Because again, this is a long axon coming in here. And that was a mistake. They come from stretch receptors in the muscle. But other than that, he can't always tell where the long connections are coming from or going to, but he can see the short connections in one region. And he can see these axons connecting directly with motor neurons. So that's why I say he was the first to see this SR connection.

And people love that because it was support for a model that had become a model of how behavior is controlled, developed much earlier. It's been around-- the idea of SR connections-- it's been around since Descartes, 17th century. And it became very popular in the next century. Whole philosophies were developed based on this idea.

And then Pavlov came along, and what did he show that made the SR model seem so much more comprehensive, even for explaining human behavior? What did Cajal discover? They could be plastic. They could change with learning. He called it conditioning. Conditioned reflexes.

The reflexologists were so happy after Cajal's discovery. Now they could explain everything. So then, my next question is, describe the argument made by Karl Lashley against the adequacy of the SR model for explaining all human behavior. I summarized it in the chapter.

And I also point out, he actually believed that when he was a student, he was studying connections and realized that, oh, if he could just get all the details of every connection in the frog, he could explain the frog's behavior. He didn't believe that anymore later. And you might wonder, well, why isn't that true?

One of the big reasons is one of those primitive cellular mechanisms we mentioned last time. Cells can have endogenous activity. They can generate their own activity. In other words, their activity isn't only determined by stimuli coming from the outside.

But then Lashley made another claim-- that a lot of human behavior, for example he used the example of playing the piano, he said the movements were so fast that reaction times simply weren't fast enough to explain them. There couldn't be a chain of reflexes to explain pattern behavior that's rapid.

Well, then what else can explain it? It had to come from a program up here that was acquired and learned in some way. Well, developing those programs, like playing the piano, it takes a lot of effort, as you know. But we know that most of those connections, they involve both neocortex and corpus striatum, especially the striatum.

So anyway, it's still a common assumption. neuroanatomists still like the SR model, even if it's inadequate. Get back to neuroanatomy here. How is the phenomenon of a retrograde degeneration used in experiments on animals to establish the existence of a major pathway taken by visual information to the neocortex? And what belief was destroyed by those-- remember what I said in there in that chapter? This is in chapter two.

I want you to know this. These have played major roles. I was reading in the 19th century literature many years ago-- I was still a graduate student-- and I read there the belief, the common belief, I was reading it, they thought the pathway from the eye-- they knew the posterior cortex was important in vision. And they knew the input came in through the eye.

They traced the pathway using just dissection methods, and it seemed to go to the superior colliculus in the midbrain. So they said, then the pathway has to go from retina to superior colliculus, and from there to the posterior cortex.

Retrograde degeneration experiments later showed that's not how the visual cortex got its information. What is retrograde degeneration? Destroy the area where a pathway terminates, and the cells where that pathway originates shrivel. They die, in some cases, or at least they shrivel. That's called retrograde atrophy or retrograde degeneration.

Keep in mind, they don't always degenerate. They often just lose weight. Sometimes they actually die over time. But where was the retrograde degeneration? It wasn't in the superior colliculus. It was in the thalamus. It was in the lateral geniculate body of the thalamus. So that was the real root, the most direct route from retina to the visual cortex, through the thalamus.

Neuro-electrophysiological methods, you should know that antidromic conduction was the major method. This is the stuff we've talked about here. What is antidromic stimulation? If you already know where the pathway probably originates-- and this is the big problem of the method, you've got to know where to look-- you record from the cells and stimulate where you think their axons are ending.

And when you stimulate, you can record action potentials from cell bodies, you can tell that you're recording from a cell whose axon ends where you're stimulating, because the waveform doesn't show any evidence of the synaptic delay, or the waveform of a postsynaptic discharge. The action potential is directly invading the cell body from where you're stimulating. That's antidromic stimulation. It was used by Sherrington and has been an important method. Even now you see physiologists using this, because they want to know something about exactly where the cells are that give rise, say, to pathways to the spinal cord.



All right. So now, tract-tracing, in answer to your question finally. What was the major difference between the tract-tracing methods of Markey, the earlier one, and Nauta, the neuroanatomist who had the last part of his career here at MIT? What was Markey's method for? It was very different from Nauta's. Nauta would've used Markey's method if it has satisfied what he really wanted to do.

The problem with it was it was only for degenerating myelin, so you could follow pathways. I read a study by Karl Lashley on the retinal projections in the rat done with the Markey method. He got most of them right, but he missed all the smaller ones.

Nauta wanted a more sensitive method, using silver methods, because he knew from a Russian technique-- [INAUDIBLE], it was his technique-- could stain even really fine axons, in even a terminal region, and even unmyelinated axons.

**AUDIENCE:** [INAUDIBLE].

**PROFESSOR:** Sorry?

**AUDIENCE:** How did he determine from looking at the Nauta tracing that there [INAUDIBLE]?

**PROFESSOR:** Oh, he didn't always get it right. But no, you're making a big assumption. But you can follow myelin fairly close to the terminals. And if he sees them going all the way and entering the superior colliculus, and that's as far as he can follow, he assumed, OK, they're terminating in the superior colliculus. And he could even see part of the layer in the superior colliculus where they would travel. He just couldn't see that they were turning up and terminating in the top layers. But he still got it right, the main projection. Same for the geniculate body, same for the pretectal area. Totally missed the one to the hypothalamus. That was even missed with most of the Nauta methods.

And then Nauta developed his method. It's a great method because it suppressed the staining of the normal axons and left the staining of the degenerating ones. He used an oxidizing agent. And the normal tissue was oxidized faster than the degenerating tissue. OK.

And then, when he came to MIT, he had a research associate named Lennart Heimer from Sweden. And he had a technical assistant, Robert Fink. The two of them each developed a more sensitive method that could stain the axons all the way to the boutons.

When Nauta first saw it, he said, this looks like measles in the brain. He saw those little dots. Could these really be boutons? I remember him saying it. Well, they were boutons, and that was verified by Heimer in the electron microscope studies.

So this is just a little about Nauta. You can read about his history and how he came to MIT as the first anatomist in a psychology department. All his students worship him. I'm one of them. He actually signed my PhD thesis. He had just come here. But he gave me some crucial help and let me do some things in his lab, and then I became his post-doc for two years afterwards.

This is an example of the Fink-Heimer method. These are retinal fibers. And this brain has been damaged right after birth on one side. And look what's happened. You see these-- I'm just pointing to areas where you can see the terminations. The axon bundles are very heavily stained. Here you see some of them crossing the midline abnormally. You don't see this in the normal lining.

So right away, we use this method to make discoveries about what happens after early brain damage. Think of cerebral palsy kids. They have early brain damage. What's happening to them? Why is some of their behavior so abnormal? They have abnormal brain connections. We discovered them in the visual system of the hamster initially. They've been discovered in many other animals since. Here you see some of them are terminating actually on the wrong side of the brain. And actually, that causes abnormal behavior.

Now, what about HRP, another early method? What does it mean? Horseradish peroxidase. It's a plant enzyme that when you inject, you inject into an area of axons. It's taken up by the axonal endings, endocytosis we call that, cell drinking.

They take it up, and they get encapsulated in little vesicles and transported back to the cell body. That's retrograde transport. If you inject the HRP in a region of cell bodies, it gets taken up by endocytosis in the cell bodies. It gets incorporated into vesicles and gets transported all the way down to the endings. That's anterograde transport.

So you have to learn to separate those two directions, but it's a wonderful tracer. Now we're using more fluorescent molecules that have the big advantage. You don't have to stain them. You just prepare it properly, put them on slides, and look at a good fluorescence microscope, and you can see the fluorescent molecules.

So let's show you those things. Here's an HRP method used in a developing hamster. I'm using dark field microscopy here. And so the HRP is showing up. It's very bright on a darker background.

And here you see something unusual. You don't see this in the older brain. Some of the axons are going right into the somatosensory nucleus more immediately. And those disappear with development.

Here is a retrograde tracer. And here, we put the tracer in the superior colliculus of the midbrain, waited a little while for the retrograde transport to take place. And you should know the molecule involved here. We used kinesin and dynein. For the two directions of transport. There are molecules that-- they can grab the protein and latch onto the structures of the microtubules and move the substance along.

But here they've reached the retinal ganglion cells. We've flat-mounted the retina here. And here you see these bright cells in the retina. We even see some of the axon coming in and assume their dendrites. Those are the cells that are giving rise to the axons we've labeled back in the midbrain, 17 millimeters away, in the hands of the adult hamster.

Here you see the same thing in the retina, but with a different label. It's called nuclear yellow. We didn't have to do any staining, but we did for these cells. That's HRP. We've used two different retrograde labels that we've injected the label on two different sides of the brain and shown that there are a few cells in the retina whose axon branches, one goes to one side, one goes to the other side.

And here, another double labeling experiment. Here, there's a bunch of cells with fluorogold. The label was put in the superior colliculus, and we put the other label, little plastic beads containing a substance, we call them the red beads, they also get taken up, and they fluoresce-- red, as you can see.

So with a different wavelength of illumination, we can see that label. And you see that these two cells are double labeled. I'm taking the same field of view with two different wavelengths of light. Beautiful advantage of the fluorescent methods for retrograde tracer. So these two cells project to both the ventral geniculate body and the superior colliculus, the rest of them only to the superior colliculus.

And then we use cholera toxin as this-- you say, why would you use such a poisonous thing? Well, it's a modification of cholera toxin, so it's fairly safe. I used to tell my students that half the substance you see on these shelves are toxic, so I want you to wear gloves, wear a mask, and don't spill things. And if you do, clean it up properly.

But anyway, cholera toxin, we only used a subunit of it. But when it works-- not all cells will take it up and transport it as well as other axons. But when they do, you get this beautiful picture. You get axons that look like they've been stained with the Golgi method, but you can trace them over long distances.

And here, I'm just showing it low power. From one part of the retina, you can see them reaching-- and here it is in dark field and light field, two different microscopic methods. And just one more method here-- a modern thing, *Recent Science* has a beautiful picture on the cover showing callosal axons in humans.

These are pictures. Here's a dissected brain showing fibers coming in and out of the neocortex. You can see the little bundles there. And you see some of them going right down into the brain stem. Well, here they are in a living human brain. They didn't inject any tracers.

They've worked out the methods to map the major directions of water diffusion-- where the water is moving. It tends to move, of course, down axons and not across axons. So you set the computer, say, to start here. And it will follow the bundles of axons from that region as far as you want.

So they traced them here through the-- we call it the internal capsule-- you'll learn all about this soon-- and into the brain stem. And then you can see, from different areas of the cortex, they traced them going different ways.

It made a mistake here. It traced them into the cerebellum, and there aren't any connections like that. So depending on how the software is working, it can jump to the wrong bundle. That's one of the problems.

The other problem is it's not actually tracing connections. Remember that. It's just tracing the large axon bundles. So when you see a study advertising, new connections seen in the human brain found with diffusion tensor imaging you know that it's a great overstatement. They've not seen connections. But it is a beautiful method to see the major pathways. And we've been able to confirm major pathways seen in the monkey and experimental methods. We now can see them in humans. And sorry I took a little extra time, but we got through all these pictures.

So think about it. Look at the chapter. Come up with your questions if you have any more questions, because we'll be going on to chapter three next time.