Hypothalamic Development and Sexual Differentiation

Stuart A. Tobet
Colorado State University, USA

I would like to thank the organizers very much for allowing me the opportunity to share with you some stories this evening on what you could consider to be the front end of the story that you have listened to for the last hour from Dr. Sakuma and Dr. Pfaus.

Hopefully I can get you to change perspective a little bit. What I would like you to do is to think of yourself as a cell, or a neuron in the brain of a very early developing animal, and think about over the next 25 minutes the kinds of choices that you are going to be forced to make to go from where you were born to where you are in a fully developed animal to be a part of the nuclei that Dr. Sakuma or Dr. Pfaus have talked to you about already. In a somewhat very personal trip, it is a little bit like thinking about going from the United States to Japan in terms of how far some neurons have to travel in order to find their place in the brain.

As you have been introduced to over the last hour, there are many sex differences in developing and adult brains of many different species. In fact, what is shown here on this slide are examples taken from the rat, a ferret, a guinea pig, and a gerbil, all showing different groupings that appear different on the left hand side, in each case the female, and on the right hand side, the male.

We circle those as individual groups of cells, but what they really are in reality are images taken from slides like this where you can see a slightly denser accumulation right here and a denser accumulation over here, where each black dot in those pictures represents a single Nissl-stained cell. That is, if you are going to put yourself in the frame of mind of being a neuron, you are one of those black dots right now. And the question you have to ask yourself is what are the things that control your being in this group of cells or the lower group of cells or out further in the tissue.

It is not only that you are a neuron in one of those four species I just named, but you are just as likely to be a neuron in a human compared to a rat. And the nucleus that Dr. Pfaus told you about, INAH3, is located right here in this 3D reconstruction done by a collaborator of mine Bill Byne, who actually was the 3rd person in the world to replicate the finding of the size difference between male and female brains. So on the left in each case is the male data on the right is the female data.

To go back to the question at the end in the discussion in the last period, this bar right here represents the size of INAH3 or the number of neurons in INAH3 in a human homosexual male, and you will notice, contrary to reports from Simon Levay in the early 90s, there is absolutely no difference in the number of neurons that exist between heterosexual males and homosexual males. There is a slight tendency for a difference in the volume that is taken up for that space, and that is something that we can think about. It is not statistically significant, so one would have to a priori be looking for that to believe that it is a significant difference.

Again, go back to the theme that they both have mentioned earlier, it is the aromatization from testicular androgens to estrogen that help put these sexually dimorphic regions into male brains to a large extent, or take them out of female brains. Every species more or less has a period during which there are higher levels of androgen in the male brain or in the male circulation than there is in the female.

This is actually data taken from ferrets. The one thing to keep in mind in terms of endocrine disruption, and I will not really talk about it more than just mentioning it now, is that the estrogen story, the aromatase hypothesis, is a non-primate story to a large extent. There is exquisite evidence that androgen receptors contribute specifically and very strongly to primate sexual differentiation, and it is

not that there is no androgen receptor contribution to the rodents. It is just that we know more about the estrogen receptor contribution.

There are 4 mechanisms that one must keep in mind when you are sitting there in your chair pretending you are a neuron in deciding where you want to be or what you want to be when you grow up, and that is, you have to think about neurogenesis, or being born, you have to think about getting to where you want to be, whether or not you are going to live or die on your way to that location, and what you are going to be when you grow up in terms of what you are going to make; are you going to make a particular peptide or particular receptor or what kind of characteristics? To take these things one at a time, one of the ways that people look at cell death during development is to look at apoptotic cell death, and from the perspective of neurons, apoptotic cell death is easily visualized using a method called TUNEL, which end labels degenerating DNA fragments.

This is part of a study done by Joong Park, who was a student with Michael Baum and I several years ago. He looked at cell death in ferret brains and found basically very little in the way of sex differences between male and female ferrets at a point in time when we know these nuclei were developing. In rats, there are several laboratories' worth of data that showed that there is significant sex difference in cell death, but that cell death period occurs after there has already been a significant formation of sex differences and structure.

Joong Park for another part of his thesis also looked at galanin expression, galanin being a fairly sizable peptide, shown here in neurons taken from the region in the ferret brain that I showed you before is sexually dimorphic. This is taken from a male ferret brain, whereas this is taken from a female ferret brain in the same region, and you can see there is a huge difference in the amount of galanin that is made in the same place in these two different brains. I do not have time to show you all of the data that he produced in this experiment, but to a very, very strong degree, this was completely dependent upon prenatal testosterone propionate, which, based on everything else that we will talk about, was likely due to its aromatization to estradiol.

What I will spend most of my time talking about is the issue of cell migration and cells being born and leaving the place of their birth to come and reside where they want to be. What this cartoon simply shows you is that neurons, after they go through their cell cycle, eventfully get to G1 and then leave the proliferative zone and migrate out into the tissue.

Cell behavior is very different when you think about peripheral organ generation. The liver does not have to worry about bringing its cells from a place at the bottom of the screen to a location at the top of the screen.

One of the handy things that we have available to us to mark the locations of cells is bromodeoxyuridine, which is incorporated during S phase of dividing cells, and helps us follow cells after their terminal mitosis. That is because if a cell incorporates bromodeoxyuridine during S phase, goes through the cell cycle but does not leave, but then reenters the cell cycle, once it divides a second time and a third time or a fourth time, that label, the bromodeoxyuridine**,** is no longer present, and so you no longer see those cells.

To go back to why migration is so important from my perspective for sexual differentiation and what I would like to convince you of tonight, here is some data that we produced in the late 80s with an antibody that we generated in a selective screen that recognizes this particular strange staining process.

This process, which starts in the ventricle, there is a ventricle in the middle of each panel here, and stretches out almost to the pial surface which you do not see very well in this particular image, is a fiber that is called a radial glial fiber. A radial glial fiber for neurons is like hanging on to the monorail if you are at Disneyland. Neurons will hold on to that, at least the assumption has been for many years, so that they can get to where they want to be.

What is unique about the set of pictures that you see here is that the top picture is actually taken of immunoreactivity in a female brain, the middle panel is taken from a male brain, and the bottom panel is taken from a female brain, but one that was exposed to excess testosterone during embryonic development. These are all taken from rats. What you see on the right is simply the quantitation of that data, the female on top, male in the middle, and androgen treated female on the bottom. It is not quite so simple to assume that androgen does this all the time; males are always bigger than females. As Dr. Pfaus showed you, the rules change in different places. What I am going to tell you is that the rules change at different times.

In fact, this is embryonic day 18 for the same region in which males actually start out much greater than females, they end up decreasing steadily as they age. Females have more or less always stayed somewhat high, and that is what generates this difference at postnatal day zero. So the timing of exposure to gonadal steroids might make a very large difference for what endocrine disruption you might get by exposure to particular hormones at particular times. I wish I could tell you we cloned the gene, but we did not. The gene that we think is responsible for this immunoreactivity is actually this 195 kilodalton protein. We think it is that because testosterone propionate caused a selective decrease in the immunoreactivity of that band on the Western blot.

I am going to take you through 3 different types of information that talk about differences in cell position as a way to look at sexual differentiation and putting cells in nuclei within the hypothalamus. BrdU incorporation I have talked to you a little bit about as a method. I will show you some data on live observation and why we think that is a very important way for looking at endocrine effects in development, and then a little bit on the position of cells based on their phenotype, some data of which Dr. Sakuma already introduced you to with estrogen receptor β earlier.

I showed you radial glial fibers that looked very different in appearance between males and females. I just want to emphasize with this picture here that that difference really was only in immunoreactivity. It was in the presence of an antigen recognized by an antibody and not based on the presence or absence of the actual fibers themselves. Males, females, all animals develop with radial glia that fill regions of their brains and help guide cells to their destinations. I will show you some data in a minute that indicates that cells read this road map sometimes differently depending on what they are exposed to.

Bromodeoxyuridine is in black in these images. These actually show you newborn cells in ferret brain as they move along radial glial guides. We have done this analysis now in ferrets, rats, and mice. And in mice right now we are completing a rather extensive study, but it is not finished. At the moment, most of the data shows that early born cells are more likely to be affected by gonadal steroids in development than later born cells.

I would like to show you more tonight in terms of actual movements that cells make in development. To do that we set out almost 10 years ago now to bring the process of creating the brain into the dish so that we could actually look at and follow cells as they go through this differentiation process. It is one that involves now taking transgenic mice. The quantitative data that I will show you was from dye labeling, but the movies I will show you are from transgenic mice that have green fluorescent protein or yellow fluorescent protein incorporated into their genome under specific promoter control. We create slices that are 250 μm thick in which the normal process of development can still occur and then be able to see it happen.

In order for us to believe that what we watch could be real, we first wanted to show that the nuclear development that takes place *in vivo* also takes place *in vitro*. So this is a male and a female from embryonic day 15 in a mouse to embryonic day 17. The cell group that forms *in vivo* is one that also forms *in vitro*. This image is taken from a slice that was put into the dish on embryonic day 15,

allowed to survive for 2 days, and then still form its cell grouping that we know was not there when we put it in the dish. That gave us some confidence to believe that when we put these slices in the dish that what happens might in fact reflect ongoing processes.

What you are looking at here is a movie taken from a brain slice from a yellow fluorescent protein labeled transgenic mouse in which cells are in the preoptic area. You will notice the one shocking piece relative to all the information I have just told you about radial glial fibers and how important they are, is that these cells, if they are using radial glial fibers, they are using them more as a ladder than as a rope. That is one of the things that may be important for cells to pay attention to, whether or not they are exposed to significant amounts of hormone or not. The reason I say that is because of this experiment that Rachel Henderson did in the lab a number of years ago that shows that in this panel on the upper right, male cells or cells in males taken at embryonic day 15, no longer exposed to steroids but they were in the dish at embryonic day 15, have more horizontal movement than cells from female slices. That also caused them to move faster in the dish.

So here we have a significant sex difference in the behavior of cells in a dish where we now have control over what is happening. Again, this is still preliminary, but this is where we have begun to start teasing apart the effects of hormone on these cells in the dish, and what you will notice here is the percentage of cells moving along radial glial guides was significantly increased in the slices that were exposed to this estradiol treatment, similar to what you would expect in the male pattern where we put the cells in the dish but did not give them additional hormone. One possibility for how you get sex differences in development or how endocrine disrupting molecules might affect development is by changing the way the cells are migrating.

Another independent line of data goes to the issue of cell phenotype. With the estrogen action being central to what is going on in sexual differentiation, we can ask whether estrogen receptor containing cells provide a spatial identity for cells undergoing that differentiation.

This was an early indication in ferrets of the potential movement of identified cells. This is estrogen receptor α at embryonic day 30 which is the beginning of the point where differences start to emerge in ferrets. You see increasingly more lateral expression of the estrogen receptor, which was the first indication to us that maybe estrogen receptor position might be important for trying to interpret sex differences.

I am going to show you mouse data for looking at sex differences in cell position. But to do that, I am going to take advantage of an animal that is devoid of steroidogenic factor 1. There is a little bit more to this story that I will get to in a little bit, but what I want you to remember for the next 2 slides is that steroidogenic factor 1 animals fail to develop gonads and adrenals which make them a model for embryonic gonadectomy.

The thing to keep in mind for later is that the only place in the brain that this molecule is expressed is the ventromedial nucleus of the hypothalamus that Dr. Sakuma introduced to you before. With the knowledge that the knockout for steroidogenic factor 1 is a gonad deficient animal, what you are seeing here is estrogen receptor β in wild type males, wild type females, and male knockout animals.

What we have done in looking at the positions of cells in the developing brain is to try to map everything from a central midpoint. So this is the midpoint to us, and we measured the distance that cells are located out laterally and we measure the distance from the base of the brain.

If you do that to these three images and groups of animals from those three images, then you get the following data. You get significantly more receptor in the male ventrally, significantly less dorsally, and the opposite pattern in females. So the location of the cells is actually different, very similar to the anteroventral periventricular nucleus data that Dr. Sakuma showed you. The same thing is somewhat true laterally, from medial to lateral, if you go out in columns from the ventricle there are more cells

estrogen receptor β positive in males closer to the ventricle, whereas there are more cells lateral in females.

The key question and why we were so glad to see Dr. Orikasa's paper in the spring, was that there is significant functional consequence for a cell to be out of position. As he described the data to you earlier, I will not rehash the data, but it is very important to note that if a cell is out of position then it really does make a difference for the animal's adult function.

We have not only done this for ER β which is shown here in this blue and this red bar so male would be here, female here, but also done it for two other markers of neuronal identity, $GABA_B$ receptors and $GABA_A$ receptors, two different subunits. And again, the different locations within the preoptic area are something that appears to be a sexually dimorphic characteristic.

How can steroid hormones cause these changes in development? What might they actually act upon as a substrate? There are definitely specific target genes that might play such roles.

One of them I already introduced to you is steroidogenic factor 1. In steroidogenic factor 1 knockout animals which I told you have expression only in the ventromedial nucleus, you will notice in this slide taken from Greg Majdic's work in Keith Parker's lab in Texas that there is no appearance of the ventromedial nucleus in this animal.

You can ask when you see this image, are the cells missing or are they misplaced. So what Tammy Dellovade did in my lab several years ago was look at estrogen receptor α expression in the ventrolateral quadrant, and you will see in the wild-type that it can be mapped again in that column kind of analysis as in this lateral position. If you look in the knockout, the cells are located more medially, right along here. So it looks like they are out of place.

Keith Parker's lab most recently created an animal that not only was a knockout for steroidogenic factor 1, but he replaced a gene that is driven by the SF-1 promoter; it is green fluorescent protein. What you are looking at here in a wild-type is expression of SF-1 driven green fluorescent protein that looked at immunocytochemically in wild-type. In the knockout animals, these cells are now spread throughout the region. So it looks as if, from this perspective as well, the position of cells in this region is misplaced due to the action of one specific gene.

We are beginning to do these studies now so that we can actually look by video microscopy. What you are looking at here is some of the SF-1 GFP animals where you can see cells in the ventromedial nucleus. As they move around, they tend to move more medial-lateral than some of the other cells, say these, which are actually labeled with yellow fluorescent protein under the control of a Thy-1 promoter.

The ventromedial nucleus in this image is actually right here, and in this location you see a lot of cells that are moving along the ventricular zone, following the idea that some cells know how to pay attention to radial glial fibers as ladders rather than ropes.

In terms of molecules that may play a significant role under the control of either the SF-1 gene, which is a nuclear transcription factor or other factors in development that might work in nuclei, GABA is a significant factor that might end up being an end product inhibitor.

It is interesting from the perspective of endocrine disrupters that a lot of pesticides in fact serve a dual purpose; several of them are estrogen receptor antagonists or agonists and at the same time they actually can work at $GABA_A$ receptors.

In this particular case we know that cells in the region of the ventromedial nucleus are significantly more likely to move in the presence of a $GABA_A$ antagonist, and if you give a $GABA_B$ agonist, they are significantly less likely to move, which indicates that GABA might play a significant role in this region.

So why would I even begin to experiment with GABA? That is because of this picture on the left right here, which is the normal pattern of GABA expression in a developing region of the ventromedial nucleus. It is excluded from this region of the ventromedial nucleus, and only later in life do the fibers

innervate this particular region. In the SF-1 knockout animal, the region becomes full of GABA, and we might end up seeing that as the cause of the significant difference in locations of the cells in this region.

To this point, to summarize what I have told you, a critical aspect of hypothalamic development is placing cells in their correct position. Gonadal steroids and sexual differentiation may help determine the placement of cells in positions within the hypothalamus, something that we are actively looking at strongly right now using the *in vitro* technology.

Key genes such as SF-1 may actually help determine the placement of those cells and the formation of nuclei within the hypothalamus and may be targets for endocrine disruption.

Finally, GABA is one of those signaling molecules that may provide positional information from cell to cell and be a part of the readout of what genes like SF-1 do to help translate those effects.

I will end by thanking a long list of collaborators for supplying mice of different knockouts that have helped form the basis of our interpreting early developmental events.

In my laboratory I have shown you data from Aline Davis, Margaret Van Doren and Tammy Dellovade this evening, Heather Walker and Marianne Seney, and importantly for the early work I have showed you, Mike Baum and Joong Park for the ferret data and different mechanisms; Bill Byne for the human data on INAH-3, and Keith Parker who supplied all of the animals relying on SF-1 knockout and transgenics. With that, thank you very much for your attention.

Q&A

Sakuma: Thank you Stuart for showing lots of beautiful morphologies. Dr. Tobet's presentation is now open for questions. Any questions or additional comments? Please come forward.

Q: I just wonder with the time-lapse photography, what is the time going on by these cells moving?

Tobet: Each frame is 5 minutes apart, and each of the movies that I showed you was about 3 hours. The average speed of migration for cells in this part of the brain was in the early slide about 20-25 μ per hour. In the males they were moving a little bit faster.

Q: Is that determined by developmental stage, or were you just looking at one developmental stage?

Tobet: Cell migration in the hypothalamus takes place over the period from when cells go through terminal mitosis about embryonic day 11, and there are probably still some cell movements postnatally. Nobody actually knows if the speed changes when you get older. In fact, these are the only data I know of that deal with actual migration in the hypothalamus by actually watching it. We have looked at several different ages, but at no point have we seen a different speed yet.

Q: May I ask another question?

Sakuma: Yes, please, go ahead.

Q: How long is that slice alive and well in your dish?

Tobet: It will actually stay alive for a very long time. We do not trust the cues for migration beyond about 3 days. So what happens is that they are very healthy, and neurite outgrowth comes out of the slices as Dominique Toran-Alerand showed years ago. That plethora of fibers we consider to be confusing for the cells. The slice prep that we use keeps the right thickness so that cell movements tend to mimic what happens to the best of our ability *in vivo*. So after 4 days or 5 days it probably gets a little bit different.

Q: I have just one question. Your SF-1 knockouts, their hypothalamus is obviously very different. What does their sex behavior look like?

Tobet: The downside of the SF-1 knockout as it exists right now is that it is an adrenal ectomy as well embryonically, so it only survives to adulthood with an adrenal transplant. The slide I showed you from Greg Majdic is the only set of SF-1 animals that have ever been grown to adulthood. So right now the plan is for Greg to actually do that experiment some point in the next year or two.

Sakuma: Any other questions? Yes.

Q: You mentioned that some pesticides could also have an effect on GABA modulation. What are some of the pesticides that have this effect and also have you seen any evidence that suggests that they might have an effect on people at the levels that they are being exposed to?

Tobet: The best known pesticide is dieldrin. It works in both receptors.

In terms of humans, the issue comes back to the one I mentioned early on, which is how much do you look for it to be androgen receptor dependent as opposed to estrogen receptor dependent. Then it goes back to the endpoint that Jim was mentioning; what do you actually look for in the human population?

The brains that Bill Byne collected so that we could look at human male, female, and heterosexual and homosexual brains took more than 3 years to accumulate just the number we used for that study.

Q: How many was that?

Tobet: In the controls it was 26 heterosexual males and 14 homosexual males.

Q: Thank you.

Sakuma: OK, thank you Dr. Tobet.