Sexual Differentiation of Sexual Behavior

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I'd like to talk today about some observations that are forcing us to reconsider the nature of sexual differentiation of the brain and its relation to sexual behavior. The classic experiment on this topic is actually the first experiment ever done in modern behavioral endocrinology. In 1849, Arnold Berthold castrated male chicks and examined the effects on their anatomy and physiology. It was known that, instead of growing up to be big roosters, castrated males would grow up to be very female-looking capons that provide a more tender meat than a male rooster. More importantly, capons did not display male sexual or social behaviors. Berthold surmised that the lack of glandular secretions from the testes led to this demasculinization. He tested this by castrating chicks but reimplanted the testis into their abdominal cavities. That manipulation allowed the chick to mature into a normal male rooster. Thus, as long as the castrated testes had been reimplanted and reinvigorated with blood, androgens could be released to masculinize the phenotype. Furthermore, it did not matter whether it was a chick's own testis, or some other chick's testis. Similar masculinization occurred when two male chicks were castrated and reimplanted with each other's testis. Not only did they show a change in physical morphology, but also in behavioral morphology. The reimplanted animals engaged in all the social and sexual behaviors that normal, intact male roosters engaged in. Animals that remained castrated did not.

This is a classic example of the relationship between sexual differentiation of gonads and behavior. If you are female you have two X chromosomes. If you are a male you have one X and one Y. On the Y is a gene, the testis determining factor gene, or *Sry*. This gene makes a protein, known as the HY antigen, which differentiates what would be nature's default of an ovary into a testis that secretes androgens and a protein known as the Mullerian Inhibiting Factor. This protein inhibits the development of the female (or Mullerian) ductwork, which allows the male (or Wolffian) ductwork to develop in its place. Without this action, the female phenotype develops Fallopian tubes and a uterus; males do not. Instead, males develop their own internal glandular structure. Most importantly, the testes secrete testosterone and other androgens. The female ovaries do not secrete androgens or estrogens until puberty. As a result of that circulating testosterone, what would have been a clitoris and labia become a penis and scrotum. Once born, it is usually the case that the gender of babies can be identified on the basis of their external genitalia. Of course, it does not end there. The differential pattern of androgen secretion causes a chronic secretion of gonadotropins like GnRH, whereas in females GnRH secretion is under the control of cyclic estrogens.

We assume that something about this process of androgenization leads to differences in sexual behavior, and we assume that these differences are hardwired, with male typical behavior characterized by mounting and female typical behavior characterized by lordosis. There are many organizational effects of androgens in the brain that may subserve this differentiation. Some of these were mentioned by Dr. Sakuma in his talk. For example, in the medial preoptic area of rats is a sexually dimorphic nucleus (or SDN) that is larger in males than females. Lesions of the mPOA eliminate mounting behavior in males. Application of crystalline testosterone or estradiol to this region in castrated males can reinstate mounting behavior. This is the third interstitial nucleus of the anterior hypothalamus in primates, again, larger in males than in females. Lesions of this region in macaques eliminates mounting behavior. There are other regions in the hypothalamus that are larger in females than males, for example in bed nuclei of the stria terminalis and suprachiasmatic nucleus. In fact, there are many brain structures that are larger in females than in males. Going back to the medial preoptic area, here you can see that the SDN is larger in males than in females. However, If you castrate the female at birth and treat her with testosterone, this nucleus

becomes larger. Gorski and others showed that these androgenized females are more likely to mount other females, and to mount quite frequently. Taken together, these processes define a dual effect of gonadal hormones in development: an organizational effect of androgens that masculinizes the brain, and an activational effect of both androgens and estrogens that activates the organized brain regions to produce sexual behavior. Thus, in the classic experiment, animals are castrated prior to a critical perinatal period and either given hormone replacement or not. Then, the animals are given the same hormone treatment as adults. To briefly reiterate what Dr. Sakuma showed, if you castrate females immediately after birth and do not replace with any hormone treatment, females as adults will still show female-specific lordosis in response to estrogen and progesterone treatment. However, if you castrate males during this critical perinatal period and do not replace the testosterone, the males will not show mounting behavior in response to testosterone treatment as adults. If castrated males receive the androgen treatment during the critical period, their brains will respond to the testosterone and they will show typical masculine sexual behavior as adults in response to testosterone. It is interesting to note that treatment of females with testosterone during the early critical period restricts the ability of estrogen and progesterone to stimulate lordosis.

To summarize, androgenization during a critical period of organization differentiates the brain and gonads. Then, during a hormone-driven activational period in adulthood, another critical period, behavioral differentiation occurs. If the animal has a male gonadal structure, then it will mount, intromit, and ejaculate in response to sexual cues and stimulation. If the animal has a female gonadal structure, then it will solicit, pace, and lordose in response to sexual cues and stimulation. Thus if the animal is an intact and nonandrogenized female, she will seek out, attract, solicit, pace, and lordose. If the animal is an intact and androgenized male, he will seek and then react to the female's attractions and solicitations. If she runs away he chases; if she holds a lordosis, he mounts, intromits, or ejaculates. If the animal is an androgenized female, then she will display more male-like behavior, with mounting and intromission patterns. Finally, if the animal is a castrated and nonandrogenized male, he might as well spend his life doing feeding studies; he will never display sexual behavior.

These, then, are the levels of determination that we believe occur: Chromosomal sex leads to gonadal sex which leads to hormonal sex which leads to morphological sex which leads ultimately to behavioral sex. This has been the dogma. It is not necessarily true. Frank Beach observed that, "females of a large number of mammalian species may under certain conditions mount other animals in a manner which to a greater or lesser degree resembles the copulatory pattern of males". These are not androgenized females. These are intact, normal, cycling females in a large number of species. What is the hormonal basis of this behavior?

I would like to discuss some work done in my lab by my former postdoctoral fellow, Dr. Paul Vasey, who is now at the University of Lethbridge in Alberta, Canada. Paul's work examines homosexual female-female mounting in Japanese macaques, also known as snow monkeys, and whether they represent a kind of natural experiment in androgenization. I would then like to mention the findings of some ongoing work in my lab by my students Veronica Afonso, Soraya Centeno, Anik Jacques, and Amelie Woehrling, on the ability of nonandrogenized female rats to mount males.

But first the Japanese macaques. Most of you are familiar with them. In one of their native habitats at the tip of Honshu, you can see them in the snow and trees. Of course like all animals that get cold, these animals love to go into natural volcanic hot vents. Sometimes they get overheated like we do, so they come off into the rocks to get cold again. They are very beautiful animals. Their sociosexual behavior is very rich. Here is a male. Here is a female grooming the male. Here is a young male playing; here are male and female together. Of course if the female is in heat during the breeding season, which is now, from November to February, the males get the opportunity to mount. Here is Paul Vasey and some

newfound macaque friends last November in another natal colony of Japanese macaques in Arashiyama. Here is a male mounting a female. Notice they are in the trees. Here is a female mounting a female. Here is a female mounting a male – the male apparently would not mount this female when she wanted to be mounted, so she may be showing him what to do. When Paul was in graduate school at the Université de Montréal, he noted that females would form stable lesbian consortships during the breeding season. That is, when they are in heat, they will pair up female to female, and mount each other repeatedly. Here is a photograph of one female mounting another female whose daughter is assuming the same posture that mom is assuming. However, the juvenile female did not get mounted. Only mom. Paul observed that there is more female-female mounting than male-female mounting. In fact these females will pair up and mount on the order of 15,000 times during the breeding season. Males, on the other hand, are lucky to mount 200 times. It could be argued that perhaps this female-female mounting is a "Hobson's Choice", that females do this because there are not enough males. Paul varied the number of males and found that the females would pair up at exactly the same rate. Paul also found that the mounting behavior did not serve any obvious reproductive or social function. The females that display this behavior do not bear more offspring and they do not appear to use the behavior to solve social conflicts, like bonobos do.

Furthermore, Paul observed intersexual competition. When a male comes upon two females, he may try to copulate with one or both. But it was often the case that he had to fight one to get access to the other. Paul asked the question; who does the other female choose? Well, it turns out that the male competitor was chosen 2.5% of the time. Other times the female chose neither, but 92.5% of the time she chose to remain with her female partner. We thought that this might represent a natural experiment: perhaps these females are more androgenized than other macaque females that do not display mounting behavior. We examined a region of the macaque brain, the dorsocentral nucleus of the anterior hypothalamus. This region is sexually dimorphic and is larger in male rhesus macaques compared to female rhesus macaques. It is a homologue of the INAH-3, based on its location and its dimorphism, but it is also analogous to the medial preoptic area because lesions to this region eliminate mounting in male macaques. We hypothesized that if the females are mounting, it could be because they are more androgenized. For this to be true, their ovaries or adrenals must have secreted androgen during a critical perinatal period. Thus, we expected that there would be less dimorphism in this brain region of the Japanese macaques compared with the same region of rhesus macaques.

We obtained brains from 8 adults and 7 juveniles, and we dissected squares of hypothalamus and overlying tissue. The tissue was then sliced into 70 micron sections, stained with thionine, and cell counts and volumetric analyses were performed under a microscope. Here is an example of the stained tissue from adult and juvenile males and females. When we looked at neuron counts and volumes, we discovered the same dimorphism that we would see in rhesus macaques. The region was larger in males than in females, and by the same ratio that has been reported by Byne in rhesus macaques. Furthermore, we noted that the juvenile males and females were not dimorphic. Thus, androgen secretion in males during puberty may be responsible for the dimorphism. We concluded from this that there is no evidence of androgenization in the female Japanese macaque, whereas there is evidence of pubertal androgenization of males that has both organizational and activational effects. With regard to female mounting, we have to conclude that the behavior is determined more by social environment and experience with sexual reward than by androgenization.

What about rats? Female rats display low levels of female-female mounting throughout their estrus cycle. It decreases when males are present, presumably because they prefer to copulate. Socially dominant females mount more frequently than subordinate females, and female-female mounting does not alter progestational state. Furthermore ovariectomized rats have been observed to display female-male mounting, but only when primed with estrogen and progesterone.

Female rats have smaller sexually dimorphic structures in the medial preoptic area than males, and treatment with estrogen and progesterone in adulthood does not alter this.

Here is an example of a female-male mounting. The female grabs the flanks, just like a male does, pulls her rump in, shows pelvis thrusting, and then dismounts. This female, unlike the ones that Roger Gorski studied, was not primed with androgen during the critical perinatal period. These are normal, gonadally intact, nonandrogenized females. So we ask the question; what subserves female-male mounting? Our first studies examined the hormonal mediation of this effect with different steroid primings: estrogen and progesterone, estrogen alone, progesterone alone, and no hormone priming. Females were given five 30-min test sessions in our bilevel chambers, and we examined their behavior during the first and last trial.

Females primed with estrogen were the ones that displayed high levels of mounting on their first and even subsequent trials. Females primed with progesterone alone did not show the behavior. Unlike female-female mounting then, ovarian hormones seem to modulate or mediate female-male mounting.

We then asked whether this behavior is altered by prior sexual experience. We gave animals either 1 or 10 prior heterosexual experiences with a stud male, or the same number with a castrated male. This figure depicts the behavior with the castrates and the behavior with the studs. There was virtually no mounting when the females had prior heterosexual experience, whereas they displayed plenty of mounting if they have experience with the castrate. So, prior heterosexual experience attenuates the proportion and frequency of female-male mounting. Prior mounting experience did not alter the frequency.

We asked if female-male mounting is due to stress. When females are primed with hormones they become sexually receptive and proceptive. They desire sex, and demonstrate this by soliciting sex at a high rate from males. Perhaps not having their solicitations result in mounting from the castrated males stresses them to the point that stress hormones such as corticosterone are elevated. From the same groups as those above, we took blood samples immediately after the first trial or 4 days after the study to analyze corticosterone levels. Notice that the females showing the most mounting between the test day and baseline are not showing any elevation in corticosterone; therefore mounting cannot be due to stress or elevated cort levels per se. Interestingly, females with a stud male show high levels of cort, as if normal copulation is stressful.

Finally, we examined sensory mediation. We asked whether somatosensory stimulation of the vagina and cervix, flanks, or olfactory stimulation might do to the expression of the behavior. Relative to rats just being handled that show high rates of mounting, vaginocervical stimulation almost eliminates it, whereas flank stimulation augments it. Removal of the olfactory bulb eliminated mounting. So it seems to be dependent on odors from the castrated males.

We then examined the induction of Fos protein, a cellular marker of neuronal activation, following female-male mounting, and the effects of lesions to activated sites or estradiol implants to these regions. The regions in question were the medial preoptic area, the ventromedial nucleus of the hypothalamus, and the medial amygdala. As you can see, they are all on the pathway that receives olfactory input and relays it to the medial amygdala, with outputs via the stria terminalus to the hypothalamus. Fos was induced in all three regions. There were black nuclear dots of immunoreactive material in the groups that mounted, but not in the groups that did not mount. This was long enough after estrogen and progesterone that the hormones are not inducing Fos themselves. Given that these regions were activated we then implanted bilateral cannula to all three regions in a different set of hormone-primed females. We delivered excitotoxic lesions to these regions using NMDA or implanted estradiol in ovariectomized animals. Lesions in all 3 regions eliminated female-male mounting, whereas estradiol implants only in the ventromedial hypothalamus restored it.

Furthermore, we know that if we pretreat the castrated males with a dopamine reuptake blocker,

such as amphetamine, we can increase the number of males that will mount on their very first trial with the females. Amphetamine sensitizes dopamine systems in the brain. Dopamine is critical for mounting behavior and other forms of forward-directed goal locomotion. When animals had the amphetamine either paired or unpaired with the environment that they copulated in, the treatment elevated mounting in the females. So it is following very similar rules to the male.

We conclude from our experiments with female Japanese macaques and Long-Evans rats that mounting behavior does not require perinatal androgenization as a prerequisite; that it serves no reproductive function and may be of limited social value. In female rats the ability to display female-female mounting occurs throughout the estrus cycle, whereas their ability to display female-male mounting is constrained to their period of behavioral receptivity. This behavior also depends on olfactory cues from the male. Somatosensory stimulation of the vagina and cervix inhibits, whereas stimulation of the flanks facilitates female-male mounting, and female-male mounting is not induced by stress or high cort levels. Mounting the males induces Fos in regions of the accessory olfactory pathway, including the medial preoptic area, the ventromedial hypothalamus, and the medial amygdala.

Cell body lesions of these regions abolish female-male mounting, but estradiol implant to the ventromedial hypothalamus, the same structure in which estradiol promotes lordosis, promotes mounting. As in males, prior sensitization of brain dopamine systems with amphetamine increases mounting behavior in the females.

So I would like to open up questions with the following: Is mounting masculine or feminine, or just plain sexual? Clearly it is not sexually dimorphic if both males and females show it as a natural form of behavior. This leads to a second question: Is the nature of sexual differentiation the ability of different genders to make a response, that is, is it a motor ability or is it a sensory-attentional difference that affects what we respond to and therefore the amount of experience we get practicing such responses? Can social tradition or proximate causes like sexual reward really supersede more innate biological predispositions? Or do these kinds of phenomena possess an equally biological impact? It's not that we discount sex differences in sexual behavior; clearly there are. But we are questioning what the nature of those differences might be. Is the nature of sexual dimorphism sensory or motor?

Finally, could endocrine disruptors, such as kepone, diethylstilbestrol, or DDT, alter sexual activity by targeting brain structures during development or even in adulthood that are critical for sexual behavior, in addition to their known disruption of endocrine organs responsible for reproductive function? The idea here is simple. If animals are producing viable eggs or sperm, they still may not copulate or show an interest in sex partners because environmental estrogens, or other environmental toxins, alter the function of critical regions of the brain. If we are trying to replenish endangered species and we are focusing our efforts on eggs or sperm, we may be missing the biggest sexual organ of all: the brain. Thank you.

Q&A

Sakuma: Thank you, Jim. Dr. Pfaus's presentation is now open for questions. Any questions or comments?

I am very much interested in that the estrogen implant in the ventromedial nucleus will cause facilitated mounting. There have been several groups which have shown that the ventromedial hypothalamus contains neurons that suppress mounting in female rats.

We have presented at oral meeting last month that the small disruption of ventromedial efferents causes female mounting induced by a very small amount of estrogen. I think you have seen our poster at that meeting. How do you think about the possibility of estrogen inhibiting VMN neurons for mounting?

Pfaus: I think that is an excellent question. The nature of estrogen's effect on lordosis is I think still an unresolved issue. If you put neural excitatory agents, for example, glutamate, in the ventromedial hypothalamus, you actually inhibit lordosis and you increase fighting behavior.

So the male may mount, and the female may even have a high level of estrous vaginal secretions that indicate to the male that she is receptive. So the male follows, he tries to mount, she rears up and fights. It is very interesting, it could very well be the case that estradiol activates neural systems that facilitate lordosis and mounting, but at the expense of systems that normally cause the female to respond to flank stimulation by fighting instead of lordosis. Thus, activation of lordosis might occur through a form of disinhibition. As this influence wanes following repeated copulations in time, the pathways for fighting are reactivated, as occurs during estrus termination. It may be the case that glutamate systems in the ventromedial hypothalamus are normally active when the female is not in heat, but are inactivated by another neurochemical pathway that is stimulated by estrogen action.

So I think we know that the prevailing theory is that estrogen facilitates the activation of the VMN and that, in turn, facilitates lordosis. It may very well be the case that estrogen inhibits the activation of a certain cluster of neurons within the VMN and that, in turn, disinhibits lordosis.

Sakuma: OK, thank you. Any other questions? Yes, please.

Nishikawa: My name is Nishikawa from Mitsubishi Chemical Corp. I have a question I'd like to ask Dr. Pfaus, but first I'd like to check something with you, Professor Sakuma. I think there is quite a difference in sexual differentiation in the brain of rats and human beings. In the case of rats, estradiol binds with α fetoprotein and does not migrate to the brain, and testosterone changes to estradiol in the brain where sexual differentiation is caused by the estradiol. In the case of human beings, concentration of estradiol increases 100 times during pregnancy and does not bind with α fetoprotein, so the high concentration of estradiol would logically transfer to the brain of the fetus. It is my understanding that testosterone therefore causes sexual differentiation to occur in the brain, is that correct?

Sakuma: I would think so. A human female having undergone testicular feminization would have a perfectly female brain. A rat or mouse having undergone testicular feminization would have a male brain, which suggests that androgen hormones have the predominant effect in human beings. In the case of congenital adrenal hyperplasia, for example, androstendion is extremely high. You can see that the child has been masculinized if you have them draw pictures or observe the way they play. One current possibility is that, in the case of human beings, perhaps androgen causes sexual differentiation not through estrogen receptors. Another possibility is induction of aromatase

depends on estrogen, so with TFM, this can be interpreted as there being a lack of estrogen reaching the brain. Because experiments cannot be conducted using human beings, you can look at a female child with excessive male hormones in the case of congenital adrenal hyperplasia, or a male for which feminization has occurred because of Predar Disease, whereby synthesis of all steroid hormones decreases and both female and male hormones decrease. The facts that hormones are doing something are in epigenetic condition is shared by both rats and human beings. I deliberately wrote "aromatase hypothesis" on my slide for rodents, but it does not apply to human beings. That is what I think.

Nishikawa: Thank you very much. I would next like to ask Dr. Pfaus a question. Experiments cannot be conducted using human beings, but what about monkeys? According to what I have studied, sexual differentiation occurs in the same manner in both monkeys and human beings. Could you tell us what you think about this?

Pfaus: In terms of sexual differentiation of the brain, monkeys and humans are very, very similar. In terms of behavior, it is actually very difficult to see a sexually dimorphic sexual behavior in humans, unlike monkeys. Who is on top, who is on the bottom… these seem to be culturally and experientially determined.

There are many sexually dimorphic structures in the human brain. Here you will notice the corpus callosum and anterior commissure. These two structures connect the two hemispheres of the brain, and they are always larger in females than in males. That is true not only of humans and of macaques, bonobos, and chimpanzees, but it is also true in rats and lagomorphs like rabbits.

Much of this sexual dimorphism in the brain seems to have been preserved throughout evolution. To the extent that those dimorphic brain structures are mediating behavior, we then have to ask what about the behaviours is dimorphic.

Now sexual behavior in most animals seems to be dimorphic; whether animals are going to show a female pattern or a male pattern depends on a very sophisticated interplay of hormone actions during the critical perinatal period, but also on odors and social cues in adulthood. In humans, we do not really have a sexual dimorphism other than the one based on our anatomy. So which gender initiates sex, accepts sex, what positions we use, seem to be regulated by cultural determinants and by our own experience with sexual reward, rather than an instinctual, sexually differentiated response based on a dimorphic human brain. The link between dimorphic brain and dimorphic behavior seems to break down at the level of humans, at least with regard to sexual behavior.

Sakuma: I believe that interpretation is rather new. Any other questions? Yes, go ahead.

Lawton: Hi, I am George Lawton with the Endocrine Estrogen Letter. That was an interesting observation, an interesting theory about how chemicals might be a cause in creating this effect, or as an avenue for exploration in animals. And I was wondering; do you think any of this research suggests that homosexuality among either males or females in humans might be an endpoint of research for endocrine disrupters and endocrine disruption research?

Pfaus: I suppose, but only if you regard homosexuality as something maladaptive. I do not think the current prevailing theory really regards it as that. And besides, homosexuality existed long before there were environmental estrogens.

One could argue that there may be some environmental estrogens that have seeped into the water table in Arashiyama. This would open up the possibility that the expression of female-female mounting in the Japanese macaques there is due to some abnormally high level of environmental estrogen. I think it could be an endpoint, but again we have to ask is this

an abnormal behavior. Certainly homosexual men and women are perfectly capable of producing offspring. The fact that they do not show the same partner preference, the same sex reward preference in terms of the characteristics that they desire in a mate, is probably variation, not only in our species but in plenty of other species, rather than something abnormal.

Lawton: Thank you.

Sakuma: Thank you, Jim. Time is up.

Q: Hello, I would like to ask one more short question to both of you. I see that you both use Long-Evans rats. Do you have any reason for using Long Evans rats for these kinds of behavior tests?

Sakuma: Well, they are more sensitive to odors than Wistar or Sprague-Dawley. That is the reason I use Long-Evans for odor preference. Is that OK?

Pfaus: I use them just because they are pigmented, and pigmented rats have more of the “real” rat genome in them. Their behavior is much more vigorous and as Dr. Sakuma said they are more responsive to odors than the non-pigmented animals are. They show odor induced erections, whereas the non-pigmented animals do not. So there are lots of reasons to use them given how normal they are relative to how less responsive albino rats are.

Sakuma: Thank you. Now I will call Dr. Stuart Tobet to present on morphological sexual differentiation of the hypothalamus.