Estrogen Receptor and Brain Sex Differentiation

Yasuo Sakuma
Nippon Medical School, Japan

The objective of this evening session is to discuss process of sexual differentiation in animal models. I've been told to strictly observe the schedule but in the first place I would like to express thanks to the Ministry of the Environment and other sponsors to have provided an opportunity to organize a session in this very important meeting. First three lectures will focus on, respectively, electrophysiological, behavioral and morphological sex differences in the rat brain. Professor Nagahama will contribute from a comparative viewpoint, describing his recent important discovery of sex-determining gene in a model fish, Medaka.

My talk will forms on electrophysiological characterization of newel circuitry in the rat brain which regulates female rat sexual behavior under the effect of estrogen.

From a viewpoint of a physiologist, female and male animals are distinguished based on two characteristics. The one is in reproductive endocrinology. With certain exceptions of seasonal bleeders and reflex ovulators, female mammals, in general, repeat reproductive cycles. The cyclicity is a result of positive feedback action of estrogen on the brain, which does not work in the male. As a result, males show constant estrus.

Secondly, many aspects of reproductive behavior are sexually dimorphic. In the rat, maternal, solicitatory and, in particular, lordosis reflex, is peculiar to females. Male rats are territorial, and attack conspecific males intruding their territory. Mounting characterizes male sexual behavior. In both female and males, hetero sexual preference, is shared in their sexual interactions.

The behavioral sex is determined, in the rat, during their perinatal period, regardless of their genetic sex, and experimental manipulation during their "critical period" readily alter their sexual phenotype. For example, testosterone, an aromatizable androgen, masculinizes resulting masculine endocrinology or behavior. In females or neonatally castrated males, female features develop in the absence of aromatizable androgen.

The movie shows male and female interactions between a Long Evans hooded male and an albino female. The male mounts the female with his paws pressing her lump. In response to the tactile stimuli, the female shows the lordosis reflex, a dorsiflexion of the vertebral column. The lordosis reflex can be evaluated by either its frequency or strength. The two usually parallels, and both provide reliable measure on its receptivity. In males, mounting activity can be, evaluated based on frequency or latency, this time required for its onset.

The other movie shows experimental reversal of sexual behavior by androgen treatment to females and neonatal castration of males, both were accomplished when they were born, namely during the critical period. Note rat only lordosis and mounting, but other components of sexual behavior, which we call solicitation or provocation, have been also reversed.

The masculinizing effect of aromatizable androgen has been explained by an "aromatase theory" in the rodent. During the late fetal and neonatal period, which spans day 18 of pregnancy and neonatal day 5, fetal liver secrete an estrogen-binding gamma globulin. The protein binds to circulatory estrogen and prevents its entrance to the brain. This estrogen of material origin or placental origin does not cross the blood brain barrier. In female neonates, the brain develops in the absence of estrogenic effect. In males, testosterone is produced in large amount during this period. This steroid is not bound by estrogen-binding protein, and enters the brain without any hindrance. Aromatase, which produces estradiol from testosterone, or estrone from androstendione, is abundant in neurons in the hypothalamus and limbic

structure of the brain, which are involved in the regulation of reproductive endocrinology and behavior. The product, estrogen, through nuclear or here-to-fore unknown membrane receptor, regulates genetic regulation of neuronal growth, synaptic formation, myelination and causes masculinization of the brain. The problem is this elegant system is, once the estrogen-binding protein is saturated by an overdose of estrogen, it can get ready access to estrogen receptor, other non-steroidal molecules with estrogenic activity are not recognized, as in the case of diethylstilbestrol(DES), and enters the brain and binds to estrogen receptor, presumably estrogen receptor α.

A peculiar characteristic of estrogen receptor molecule is, its expression is regulated, during either the period of sexual differentiation or adult life, by its ligand. This picture shows that both in the preoptic area and the ventromedial nucleus of the hypothalamus, the number of estrogen receptor α immunereactive neurons differ significantly between the sexes. Large numbers of estrogen receptor α positive neurons have been visualized in females than in males.

The ventromedial nucleus of the hypothalamus is the major target of estrogen to facilitate lordosis reflex in female rats. Electrical stimulation of this structure enhances, and electrolytic lesion of the ventromedial nucleus diminishes this reflex.

A major projection target of the ventromedial nucleus is the dorsal portion of the midbrain central gray. As in this panel, electrical stimulation exaggerates lordosis reflex, in a dose-dependent manner to the intensity of the current.

We have evaluated the effect of estrogen on this neural connection, between females ovariectomized as adults, females given testosterone during neonatal period, males orchidectomized on the day of birth and normal males. The experiment was accomplished when they were sexually matured, after checking their behavior. That is, ovariectomized females and neonatally castrated males showed lordosis when injected with estradiol whereas testosterone-treated females do not. Stimulation electrodes were placed in the central gray and extracellular action potentials were recorded from the hypothalamus under anesthesia. Note that there is no quantitative difference in the number of recorded neurons between the groups.

The measures for the effects of estrogen on neural excitability were the threshold and the refractory period for antidromic activation. The threshold is low when neurons are readily excited. The shorter refracting also shows increased excitability.

The figure summarizes the effects of estrogen on the ventromedial projection to the midbrain central gray. In groups of animals which show the behavioral effect of estrogen, lordosis reflex, estrogen significantly decreased the threshold for antidromic activation, here in the ovariectomized female and neonatally orchidectomized males.

Thus the neural circuitry that originates in the ventromedial nucleus, that project to the central gray is sexually dimorphic in terms of electrophysiological parameters. The projection changes synapse in the central gray and continues to the medulla. We have also shown that medullary projection of the central gray is excited by estrogen.

Another target of estrogen action on the regulation of lordosis reflex is the preoptic area. Because this area is penetrated by many axons of passage with origins in estrogen-sensitive brain structures, we disrupted these axons of passage by dorsal deafferentation of the preoptic area. The cut eliminates fibers from the septum, cingulate cortex and amygdala, among others. The females rats carrying the cut shows vigorous lordosis reflex after recovery from the operation, showing some inhibitory in put for the behavior has been removed. Electrical stimulation of the preoptic area in these animals, which presumably activated local neurons in this structure, causes prompt and powerful inhibition of lordosis behavior as in this panel. A similar inhibition of the reflex can be induced by electrical stimulation of the ventral tegmental area, a target of preoptic efferents.

The sensitivity of the preoptic projection to the ventral tegmental area was compared between animal groups in a similar method which was applied to the ventromedial hypothalamus projections. In anesthetized animals, stimulation electrodes were placed in the ventral tegmental area, and antidromic action potentials were recorded from the preoptic area.

In a striking contrast to the ventromedial hypothalamus efferents, estrogen decreased the excitability of preoptic efferents. This panel shows increased threshold in ovariectomized females and neonatally orchidectomized males. Estrogen had no effect on either the threshold or the refractory period in neonatally androgenized females.

These results suggest that estrogen excites neural circuitry for the facilitation of lordosis, that is the ventromedial hypothalamus efferents, and at the same time inhibits neural circuitry for the inhibition of the behavior. The effect is sex specific. The mechanism for the site and sex specificity of the estrogen action is of one our current target of research.

Single unit activities can be recorded from awake, free moving animals during sexual interactions. From video frames, we categorized female behavior into 4 classes. The female approached to the male during solicitation were mounted by males, intromission occurred and finally male ejaculates. As shown in this diagram, sexual interaction in the rat consists of 10 to 15 mounts by males, which is terminated by ejaculation.

The histogram shows preoptic neural activity in female rats in estrogen, recorded during sexual interactions with a male partner. These preoptic neurons were excited in close association with each behavior fonts.

Event related histogram revealed that these neurons can be divided tentatively into 4 groups, each associated with solicitation, somatosensory inputs, or lordosis behavior. The last group of neurons, shown here, were notable because they were silent during the female was in lordosis posture. We interpret these neurons, which may inhibit lordosis, project to the ventral tegmental area as depicted in this panel.

The second topic in my talk is on sexual preference. Sexual preference in the rat depends, largely on odors. The box was designed to determine sexual preference of the experimental animal in the central compartment. Stimulus animals are placed in the two rooms, and negative pressure was applied in the central compartment with air coming through his transparent tube from the both sides carrying odors of the stimulus animals. We determined the time sniffed by the experimental subject by analyzing video frames.

In the left panel, sexual preference of a sexually vigorous male is shown. As you expect, the male prefers estrus female rat. The preference is in sexual context, because he prefers orchidectomized male than sexually vigorous conspecific. Estrous female is preferable than ovariectomized females, and he is ventral between non-sexually active male and female. A similar pattern, but opposite direction, is apparent when estrous female rats were tested. She prefers sexually vigorous males among others.

The remarkable feature of this test is, that it is highly sensitive to hormonal condition of the animal. When experimental subjects were orchidectomized or ovariectomized, they became neutral between any stimuli. We then supplemented experimental subjects with heterosexual hormones. Female specific preference was restored by testosterone to ovariectomized females, presumably through aromatization. Estrogen in ovariectomized females also induced female type preference, as shown in the left. This observation shows sexual dimorphism in neural circuitry for odor preference is somehow different from that in the lordosis circuit. It is more dependent on circulating sex steroids as adults.

As shown in this cartoon, olfactory pathways go through several structures that contain estrogen-sensitive neurons. The medial nucleus of amygdala and the preoptic area are two major targets of estrogen action on olfactory circuitry. This, we placed selective, small lesions in these structures. The section depicts the medial amygdala, and the scheme shows outlines of the lesions.

As shown in this panel, the lesion of the medial amygdala diminished sexual partner preference of estrous females toward sexually active males. It is noteworthy that the total time sniffed by the subject remained the same as the sham operated females, indicating the lesion of the medial amygdala dose not interfere with sexual motivation.

On the other hand, lesion of the preoptic area both diminished sexual partner preference and motivation. The result coincides with our earlier observations that the preoptic area contains neurons which are excited during solicitatory behavior.

The scheme summarizes several efferent connecting of the preoptic area with different sensitivity to estrogen. For example, preoptic projection to the medial amygdala, show in blue travels in the stria terminalis, is inhibited by estrogen. These projection to the midbrain locomotor region, which is relevant to solicitatory locomotion consists of fiber, either excited or inhibited by estrogen depending on this locations with in the preoptic area.

The last topic is on the sexual dimorphism in the distribution pattern of estrogen receptor β positive neurons. Estrogen receptor β has been cloned in 1996 by Swedish group as a different molecule from the classic estrogen receptor, now termed estrogen receptor α. By non-isotopic *in situ* hybridization, Dr. Orikasa in our team discovered that the distribution pattern of ERβ positive neuron is quite different between females and males.

The sex difference in the distribution pattern could be manipulated readily by neonatal endocrine treatment. In the left panel, genetic female was modified to male phenotype by estrogen treatment, which simulated aromatization of testosterone. Neonatal orchidectomy produced female phenotype in D. We noted, there is no difference in the total number of ERβ positive neurons. The result suggests that the sexual difference might be a result of different rate of migration of neurons, which will be elaborated by Dr. Tobet in this session.

The sex difference was located in an area called the anteroventral periventricular nucleus of the preoptic area, which has been associated estrogen-induced ovulatory surge of gonadotropins. Therefore, we infused antisense oligonucleotide against ERβ mRNA and examined whether the treatment interfere with ovulation. The chart shows vaginal cyclicity in females, which were given intracerebroventricular infusion of saline, scrambled oligo as control, and antisense. The females which received an antisense oligo showed prolonged estrus as shown in this example.

Statistical analyses showed that the infusion of the antisense oligo diminished the number of ERβ positive neurons significant as in the lower panel. Successive days of estrus, which would be one in control animals, also showed a significant increase. The anteroventral periventricular nucleus, which has been reported to occupy a larger volume in the female rats than in males, is thus responsible for positive feedback of estrogen on gonadotropin secretion, which is a peculiar feature in female mammals.

The preoptic area contains numbers of estrogen receptor α positive neurons. As I mentioned earlier, the number of ERα positive neurons is the number in females then in males, as show in this panel for ERα message. A double stain for ERα immunoreactivity and ERβ message showed many ERβ positive neurons, approximately 90 %, express ERα immunoreactivity.

We then questioned roles played by ERα in sexual differentiation of ERβ distribution, and attempted to study the possibility in ERα knockout mice.

Rats and mice are different in many aspect, but we were surprised when we found out, in collaboration with Sonoko Ogawa at the Rockefeller University, that in the mouse, male expresses lot more ERβ message positive neurons in the preoptic area.

The chart summarizes that the mice and the rat is quite different in the direction of sexual differentiation of ERβ positive neurons in this particular region of the preoptic area.

Despite these unexpected results, sexual differentiation in the distribution of ERβ positive neurons

appeared to depend on ER α , because ER α knock-out males had a female phenotype.

The sex difference is peculiar to the preoptic area and not in the periventricular nucleus, which contains ER β , but without known sex difference.

The difference can be detected as early as postnatal day 1, which is established firmly during the critical period.

And this makes the last slide of my talk and I thank these people for collaboration in current and part research projects.

Thank you for your attention, and if you have any question, please come forward. Otherwise, I'll call Jim Pfaus to present his new interpretation on the process of sexual differentiation which will provide different perspective to my present, rather orthodox theory.