Roles of estrogen receptor β in the regulation of social behaviors in male mice

（オスマウスの社会行動制御におけるエストロゲン受容体βの役割）

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General Introduction

1. General Introduction

1.1. Introductory remarks

Social interaction is an essential component of our life. Interaction with other individuals is profitable for survival. For instance, living in a group is advantageous for efficient foraging and detection of predators. Moreover, interaction with an individual of same species is crucial for reproductive success. Adult animals mate with opposite-sex conspecifics, and take care of their pups. Defense of the territory, choice of an appropriate partner, and appropriate behavior toward the partner and pups are necessary for successful reproduction. Furthermore, individuals of social species including rodents, monkeys, and human, establish social relationship with conspecifics through repeated episodes of social interaction. Choosing suitable behavioral repertoire to each situation and performance of these behaviors in appropriate way are necessary to establish social relationship. Living within the social relationship is essential for survival in social animals.

I focused on social behaviors in male mice in this study. Previous studies using rodents including mice, rats, and hamsters elucidated essential roles of testosterone, one of gonadal steroid hormones, in the regulation of male social behaviors. However, underlying neural mechanism of behavioral regulation by testosterone is not completely understood. Notably, little is known about the roles of estrogen receptor β (ERβ), a subtype of estrogen receptor and one of the major mediators of testosterone action. Although several lines of evidence suggested importance of ERβ in "fine-tuning" of components of male social behaviors including aggressive behavior, social reactivity, and social information processing (Reviewed in Weiser et al., 2008; Handa et al., 2012), precise role and relative importance of ERβ in the regulation of social interaction and its neural mechanism are not well understood. In this study, I focused to investigate (1) site-

specific role of ERβ in the regulation of essential components of male social behaviors, i.e. social information processing, sexual and aggressive behaviors. In addition to these "behavioral components", I intended to examine (2) whether ERβ is necessary for establishment of social relationship in male mice.

1.2. Social behaviors in male rodents

Typically, male mice behave differently toward same- and opposite-sex conspecifics. When a male rodent encounters another male, it intensively sniffs body, face and anogenital region of the opponent. In a laboratory setting such as a resident-intruder paradigm (see 1.2.3.), aggressive behavior is often observed following to social investigation. After repeated and/or a long-term social interaction, male rodents often establish dominance hierarchy (Ginsburg and Allee, 1942). On the other hand, males show sexual behavior toward a female. In mice, females spontaneously ovulate every fourth or fifth day. Behavioral estrus, in which females show sexual receptivity, lasts about 24 hours during an estrous cycle (Tomihara, 2010). Receptive posture of a female is critical for completion of male sexual behavior even though males are able to mount to a non-receptive female (McGill, 1962). Thus, males prefer a receptive female over a non-receptive female when two females are presented simultaneously (Kondo and Sachs, 2002).

To respond properly to each of different types of opponents, males mainly use olfactory information. They judge sex, age, and reproductive status of an opponent and whether the opponent is familiar one or not. Auditory information is also used for social interaction. In rats and mice, ultrasonic vocalization is utilized during copulatory interaction, juvenile play behavior, and nursing (Portfors, 2007).

1.2.1. Assessment of social information processing of male rodents

Olfactory information from an opponent is necessary for performance of social behaviors in rodent species (Rowe and Edwards, 1971, 1972). Olfactory system in rodents consists of main and accessory olfactory systems. Traditionally, the main olfactory system (MOS) has been implicated in detection of volatile odorant molecules and the accessory olfactory system (AOS) has been implicated in pheromonal communication mediated by non-volatile chemicals. Although previous studies suggested that this classification is not definite (Tucker, 1963; Meredith, 1998), relative importance of AOS for male sexual and aggressive behavior is well established (e.g. Clancy et al., 1984).

These two olfactory systems are known to converge in some brain regions including medial amygdala (MeA) (Meredith, 1998). To investigate underlying mechanism of social information processing in these brain regions, several behavioral testing paradigms have been developed. Among them, in sexual preference tests, subject animals are allowed to investigate odors of urine, or soiled bedding from two types of stimulus animal and preferential investigation toward one of these stimuli is assessed. Sexually active males preferentially investigate the odor from receptive females compared to that from non-receptive females or males. Moreover, they show preference to a gonadectomized male over an intact male rat (Xiao et al., 2004). In sexual preference tests, abilities to discriminate two stimuli and respond to intrinsically attractive stimuli (i.e. receptive females or gonadectomized males) are also assessed. In addition, total investigation duration can be used as an index of social interest to the stimuli.

Furthermore, rodents can discriminate and memorize other conspecific individuals using olfactory information. Information whether an opponent is familiar or novel is necessary for territory defense, partner choice, and parental care. Not only recognition of a same-sex individual, but also that of an opposite-sex individual plays an important role

for successful reproduction. For instance, rats and mice prefer a novel female odor than a familiar female odor (Carr et al., 1980). Moreover, it is reported that, after exposure to a novel female odor, male mice show increased levels of risk-taking behaviors (Kavaliers et al., 2008).

Ability of social recognition and social memory has been assessed using a habituation-dishabituation paradigm (Ferguson et al., 2002). In this paradigm, subject animal is exposed to a same stimulus animal (stimulus A) repeatedly with a fixed inter-trial interval. Decreases of investigation along a repeated exposure (habituation) indicate that the subject animal is able to keep the memory of the stimulus animal A. Restoration of investigation duration upon an exposure to a novel stimulus animal (stimulus B) (dishabituation) indicates that the subject animal is able to discriminate the stimulus A and B. These social information processing is crucial for subsequent social behaviors and establishment of social relationship.

1.2.2. Assessment of male sexual behavior

Male mice show sexual behaviors when they encounter a female mouse. At first, precopulatory behaviors including sniffing of facial and anogenital region and emission of 50kHz ultrasonic vocalizations are observed. Then, the male mouse shows stereotypical copulatory behaviors such as mount, intromission, and ejaculation (McGill, 1962; Hull and Dominguez, 2007). If the female mouse is sexually receptive, she shows receptive posture called “lordosis”. Lordosis posture is helpful for males to successfully ejaculate although males occasionally show ejaculation to non-receptive female (McGill, 1962).

After completion of the ejaculation, male mice rarely copulate again for 24h, unlike male rats that show ejaculation several time in a single testing day. In addition, mounting

behavior toward another male is sometimes observed as a part of dominance behavior in mice and rats (Wang et al., 2011).

1.2.3. Assessment of social behavior between male mice

1.2.3.1. Aggressive behavior

In the article by Nelson and Trainor (2007), aggression is defined as "overt behavior that has the intention of inflicting physical damage on another individual, and the potential for aggressive behavior exists whenever the interests of two or more individuals conflict." To assess aggressive behavior in male mice, individual housing and/or co-habitation with female conspecifics are general procedure to potentiate aggression toward other males (Siegfried et al., 1981). Experimental paradigm called "resident-intruder paradigm" has been widely used. A stimulus male mouse (intruder) is introduced into a home cage of subject male mouse (resident). Aggressive behaviors by the resident are then observed and recorded. To minimize the levels of fight-back by intruder mice, they are often group-housed and/or olfactory bulbectomized. Behavioral acts such as chasing, boxing, wrestling, tail rattling, biting, and offensive lateral attack are defined as main components of aggressive behavior. Aggressive behavior can also be assessed in neutral cages. In this method, experimental mice are placed in each sides of a divider placed in a neutral cage and allowed to habituate for a few minutes. The divider is then removed and aggressive behavior between two mice is observed.

1.2.3.2. Establishment of dominance hierarchy

Male mice establish hierarchical social relationship through social interaction. Wang et al. (2011) tested social behavior of group-housed (four mice) male C57BL/6J mice and reported that a linear hierarchy was observed in 89% of the cases although a non-linear

hierarchy was occasionally observed, i.e., if mouse A was dominant over B, and B was dominant over C, then A was dominant over C. In this study, it is also reported that the hierarchy was not very stable, since the rank of each mouse often (about 41%) changed between days during 7 days testing period.

Establishment of a dominance hierarchy is not necessarily accompanied with intensive aggressive behavior. The term of "agonistic behavior" includes all interactive behavior among conspecific animals such as sniffing, grooming, and submissive behavior in addition to aggressive behavior (Scott, 1966) and is often used in analysis of hierarchical social relationship. Social dominance among multiple animals can be assessed using various testing paradigms. In some cases, agonistic behavior with direct physical contact observed during behavioral tests is used for an assessment of social dominance. In other cases, social dominance is assessed by a comparison of territorial or courtship behaviors between males. Furthermore, in the tube test developed by Lindzey et al. (1966), mice are forced to compete for occupation of a narrow tube. Social rank in the tube test is reported to be consistent with the rank measured using other test paradigms (Wang et al., 2011).

1.3. Testosterone action and estrogen receptors

1.3.1. Testosterone and its metabolites

Testosterone is one of gonadal steroid hormones classified as androgen and plays an essential role in the regulation of a series of male social behaviors. In males, testosterone is synthesized from cholesterol mainly in Leydig cells of testes. Gonadotropin releasing hormone (GnRH) induces secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. Thereafter, FSH and LH induce secretion of testosterone from the testes. Testosterone acts on androgen receptors (AR) as

its original form or as dihydrotestosterone (DHT) after the conversion by 5α-reductase. Moreover, testosterone is converted into estradiol by aromatase, a metabolic enzyme belonging to Cytochrome P450 superfamily (Simpson et al., 1994). After aromatization to estradiol, testosterone regulates organization and activation of male-type neural circuitry via estrogen receptors (ER).

1.3.2. Subtypes of estrogen receptors

Two subtypes of ERs, estrogen receptor α (ERα) and estrogen receptor β (ERβ), are well known as key mediators of behavioral regulation by testosterone. They are members of a nuclear receptor subfamily, and are ligand-dependent transcription factors (Fawell et al., 1990; Tremblay et al., 1997). After ligand binding, ERs are transported to cell nuclei and bind to target sites of DNA (Kumar and McEwan, 2012). ERα and ERβ are known to regulate a wide variety of target genes including progesterone receptor, oxytocin receptor (Young et al., 1998; Lindberg et al., 2003). Moreover, rapid, non-genomic action of estradiol via ERα, ERβ and G-protein coupled ER has been focused on in recent studies (Björnström and Sjöberg, 2005).

1.3.3. Organizational and activational actions of testosterone

Two types of actions of testosterone are well documented. One is called "organizational action" and the other is called "activational action". The organizational action is permanent and is involved in formation and development of neural networks. It occurs during the critical period in lifetime, such as the perinatal and pubertal period.

Figure 1 illustrates changes of circulating testosterone levels in lifetime of male mice. From the embryonic day 18 to the neonatal period, there is a drastic increase of circulating testosterone levels, which is called "androgen surge" (blue arrow in Figure 1). During this

period, the neural network controlling male social behaviors is masculinized and defeminized. Although both AR and ERs are implicated in this process, a pivotal role of estrogenic signaling has been demonstrated (Arnold and Breedlove, 1985). During the androgen surge, aromatized testosterone enables activation of ERs only in male (MacLusky and Naftolin, 1981) since ovaries are not active and do not secrete estradiol. Furthermore, in the pre-natal period, alpha-fetoprotein binds to estradiol originated from a mother and prevents masculinization and defeminization of female brains (Bakker et al., 2006).

At the onset of puberty, testosterone levels start to increases again and reach the adult level by the end of the pubertal period (red arrow in Figure 1). Recent studies demonstrated that testosterone during the pubertal period is also necessary for full masculinization of the central nervous system (Romeo, 2003; Sisk 2015). Male hamsters with depletion of pubertal testosterone by pre-pubertal castration failed to show restoration of aggressive behavior in response to testosterone implant in adult (Schulz et al., 2004).

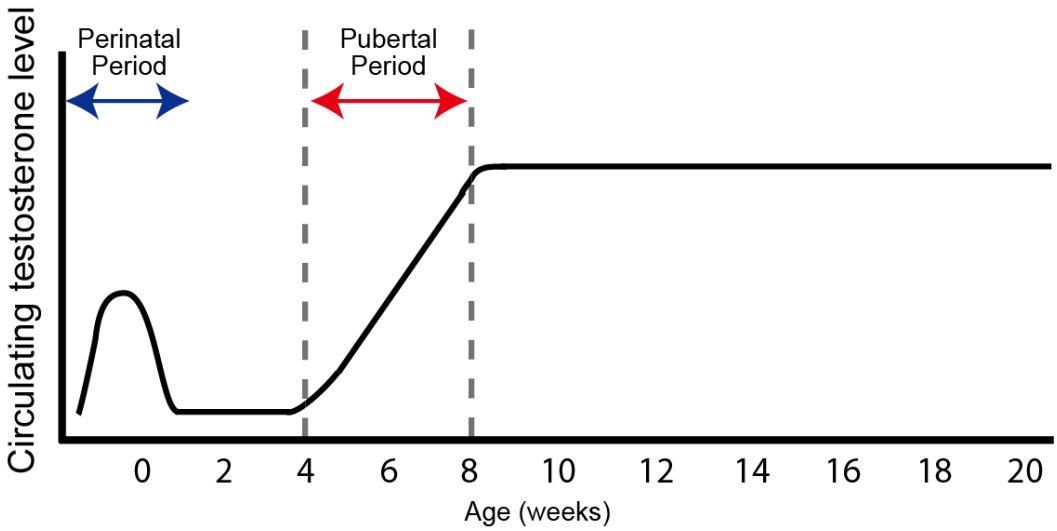

Figure 1. Illustration of the change of circulating testosterone level in lifetime of male mice. Blue arrow indicates perinatal period. Red arrow indicates pubertal period.

In contrast, the activational action is transient and occurs throughout life. Testosterone is able to fully regulate male social behavior by acting on the neural circuitry masculinized/defeminized by its organizational action. Testosterone induces behavioral and/or physiological changes through genomic and/or non-genomic action. Genomic action occurs through DNA binding of dimerized receptors and occurs after a time-lag of hours or days, is well documented as classical mechanisms. On the other hand, rapid non-genomic action, which occurs in seconds or minutes without direct binding to DNA, is also noted in recent years (Björnström and Sjöberg, 2005).

1.4. Regulation of male social behaviors by testosterone

1.4.1. Regulation of male social behaviors by activational action of testosterone

It is well established that testosterone is necessary for expression of male-type social behaviors. Gonadectomy disrupted sexual behavior of male rats and simultaneous implant of a silastic capsule filled with testosterone or estradiol but not DHT, non-aromatized androgen, can reverse the effect of gonadectomy (Meisel et al., 1984). Estrogen replacement is also effective to restoration of sexual behavior in male mice (Edwards and Burge, 1971). Thus, estrogenic signaling is important in the performance of male sexual behavior. For aggressive behavior, estrogenic signaling is also necessary (Ogawa et al., 1997) although both signaling via estrogen and androgen receptors are necessary for full expression of aggressive behavior (Nelson and Trainor, 2007). Deletion of testosterone by gonadectomy disrupts not only aggressive behavior, but also dominance hierarchy between male mice. Albert et al. (1986) reported that a gonadectomized dominant male rat without testosterone replacement lost his dominance over subordinate males. Relationship between male social dominance and testosterone level was reported in

previous studies with human (Mazur and Booth, 1998) and rhesus monkeys (Rose et al., 1971). In male mice, Zielinski and Vandenbergh (1993) revealed that treatment with physiological doses of testosterone successfully restored dominance status of males over subordinate mice treated with lower doses of testosterone.

Activational action of testosterone is also necessary for social information processing in male rodents. Xiao et al. (2004) reported that gonadectomy of adult male rats disrupted their male-type sexual preference.

1.4.2. Regulation of male social behaviors by organizational action of testosterone

The organizational action of testosterone is also necessary for expression of male social behaviors. As described in 1.3.3., estrogenic signaling in perinatal period contributes to masculinization of the nervous system. Neonatal treatment of female rats with estradiol enabled them to express male-type sexual behavior in response to testosterone injection in adulthood (Christensen and Gorski, 1978). Severe deficits of male-type sexual and aggressive behaviors and sexual preference were reported in male aromatase knockout (AromKO) mice, which cannot synthesize estradiol. Neonatal treatment with estradiol to AromKO male mice restored male sexual and aggressive behaviors in adulthood (Toda et al., 2001a, b; Harada et al., 2009). These findings demonstrate relative importance of estrogenic signaling in the perinatal period.

Importance of pubertal testosterone in the organization of the neural network for male-type social behaviors is also documented in male rodents (Romeo, 2003; Sisk and Foster, 2004; Sisk, 2015). Depletion of testosterone during puberty by gonadectomy at postnatal day (PND) 21 disrupted adult male sexual and aggressive behaviors even with testosterone complement after the end of puberty (Schulz et al., 2004). However, precise underlying mechanisms of pubertal organizational action of testosterone is not well

understood. Contribution of ERα in pubertal formation and/or development of neural network for male sexual and aggressive behavior is demonstrated recently (Sano et al., 2016) and described below (see 1.6.3.).

1.5. Neural network for male social behaviors

1.5.1 Neural network for social information processing

Processing of social information of other individual is essential to choose appropriate social behavior. As described above, there are dual olfactory systems. In the MOS, main olfactory epithelium sends olfactory information to the main olfactory bulb. On the other hand, vomelonasal organ is a receptive organ of AOS and sends information to the accessory olfactory bulb. Both main and accessory olfactory bulbs project to the MeA (Baum, 2009). The MeA has been considered to integrate olfactory information. From the MeA, the information is sent to other brain sites in the hypothalamic and limbic areas regulating male social behaviors, either directly or via the bed nucleus of the stria terminalis (BNST) (Ferguson et al., 2002). Lesions of the MeA disrupt male-type sexual preference (Kondo and Sachs, 2002). Recently, Dhungel et al. (2011) also reported disruption of sexual preference by MeA lesions. They proposed that the MeA might be important for preference exhibited by male rats toward a receptive female rat over a non-receptive female rat, but not over a gonadally intact male rat. The medial preoptic area (MPOA), a hypothalamic nucleus responsible for the performance male sexual behavior, is also implicated in male-type sexual preference. Unlike the MeA, lesions of the MPOA suppressed preference of male rats toward receptive female rats over not only a non-receptive female rat but also a gonadally intact male rat (Dhungel et al., 2011). These findings suggest that these two brain areas may be involved differently in the regulation of male-type sexual preference. The MPOA is implicated in the control of sexual

motivation (Hull et al., 1995) whereas the MeA plays an important role in processing of odor information of other individuals. It should be noted that the MeA processes not only information relevant to sexual preference but also individual discrimination. For instance, it is reported that oxytocin in the MeA is necessary for social recognition (Ferguson et al., 2001) and ERα expressed in the MeA may be involved in the control of social recognition by regulating the levels of oxytocin receptors (Choleris et al., 2003).

1.5.2. Neural network for sexual behavior

Several lines of evidence indicate the involvement of the MPOA, MeA and BNST in the regulation of male sexual behavior. Among those, the MPOA is considered to play the most critical role. Lesions of the MPOA greatly reduced male sexual behavior in rats and mice (Paredes, 2003; Hull and Rodoriguez-Manzo, 2009). An increased number of Fos immunoreactive cells were observed in the MPOA in male rats after sexual behavior (Veening et al., 2005). The MPOA receives innervations from the MeA and BNST, which are supposed to mediate information of sexually receptive female. In the MeA and BNST, increased Fos immunoreactivity was also observed after sexual behavior (Veening et al., 2005; Hull and Rodoriguez-Manzo, 2009). Ventromedial nucleus of hypothalamus (VMN) is also indicated in male sexual behavior. Involvement of ERα positive neurons in the VMN in sexual behavior is revealed by recent studies (Sano et al., 2013; Lee et al., 2014).

1.5.3. Neural network for aggressive behavior

The MeA, BNST, lateral septum (LS), anterior hypothalamic area (AHA) and VMN are implicated in the regulation of aggressive behavior. Neuronal activation indicated by an increase of Fos immunoreactivity after aggressive encounter was reported in these brain sites (Veening et al., 2005). Social odor information is integrated in the MeA and sent to the BNST, LS, and AHA. These brain sites send innervation to the periaqueductal gray (PAG), which is responsible for execution of cooperated body movement for aggressive behavior (Nelson and Trainor, 2007). Recently, an essential role of the VMN in aggressive behavior was revealed using optogenetics (Lin et al., 2011). Similar to the findings in sexual behavior, ERα positive neurons in the VMN may play a significant role in the regulation of aggressive behavior in male mice (Sano et al., 2013; Lee et al., 2014).

On the other hand, involvement of the MPOA in the regulation of male aggressive behavior is still controversial. Newman (1999) proposed a relatively minor role of the MPOA in aggressive behavior since Fos expression was unaffected after aggressive encounter in male Syrian hamsters (Kollack-Walker and Newman, 1995). However, other studies using male rats and mice indicated that the MPOA might also be involved in the regulation of male aggressive behavior (Patil and Brid, 2010; Wu et al., 2014).

1.6. Regulation of male social behavior by estrogenic signaling in the brain

1.6.1. Distribution of estrogen receptors in the neural network for male social behaviors

Both ERα and ERβ are widely expressed in the brain sites of the neural network for male social behaviors. In the hypothalamic and limbic areas, distribution of ERα and ERβ is often overlapped, but in some of areas, either ERα or ERβ is predominantly expressed. Figure 2 shows representative expression sites of ERα and ERβ in the mouse brain (modified from Mitra et al., 2003 and Handa et al., 2012). Among expression sites, the

MPOA, BNST (left panel) and the MeA (right panel) express both ERα and ERβ abundantly. In the MeA, co-localization of ERα protein and ERβ mRNA is reported (Shughrue et al., 1998). On the other hand, ERβ but not ERα is expressed in the PVN (right panel). Moreover, in the VMN, ERα but not ERβ is abundantly expressed (Shughrue et al., 1997; Mitra et al., 2003; Merchenthaler et al., 2004).

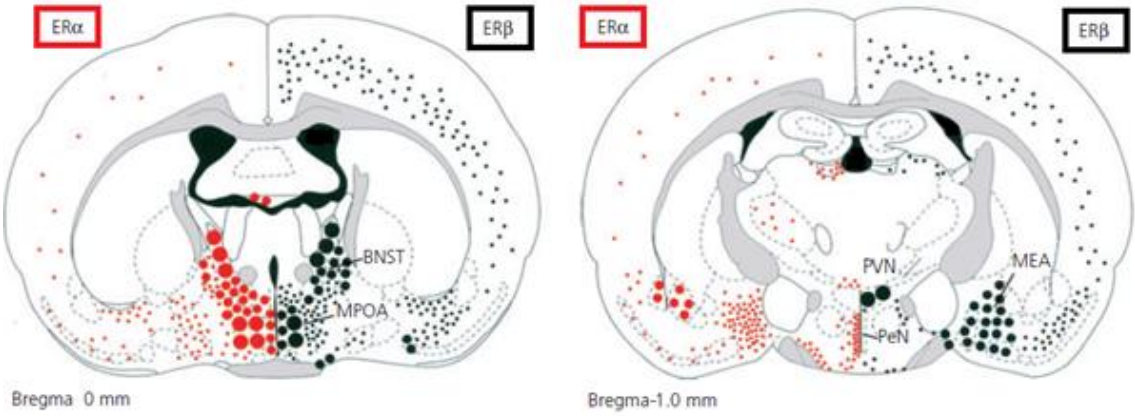

Figure 2. Differential distribution of ERα and ERβ in the mouse brain. Two coronal planes through the brain (left panel: at bregma, right panel: at -1mm to bregma) show the anatomical distribution of ERα (left, red dots) and ERβ (right, black dots). BNST, bed nucleus of the stria terminalis; MPOA, medial preoptic area; MEA, medial amygdala; PVN, paraventricular nucleus of the hypothalamus; PeN, periventricular nucleus. Gray shading shows white matter tracts (modified from Mitra et al., 2003 and Handa et al., 2012)

1.6.2. Regulation of male social behaviors by testosterone via estrogen receptors

The exact receptor type(s) and their expression site(s) those mediate activational and organizational actions by testosterone for the regulation of male social behavior are still not completely understood. Studies using knockout mice indicate that ERα and ERβ may play different roles. ERα is necessary for performance of male social behaviors since

αERKO male mice showed severe deficits in sexual and aggressive behaviors (Ogawa et al., 1997, 1998, 2000). On the other hand, the role played by ERβ in the regulation of male social behaviors is still unclear. Survival of sexual behavior and partially increased aggressive behavior in βERKO male mice suggest that ERβ may play a role in fine-tuning rather than induction of male social behavior (Ogawa et al., 1999; Nomura et al., 2002). Moreover, both ERα and ERβ are implicated in social information processing, but responsible brain site(s) may be different (Imwalle et al., 2002; Choleris et al., 2003; Kavaliers et al., 2004, 2008).

1.6.3 Site-specific regulation of male social behaviors by estrogenic signaling

It is considered that each brain site in the neural circuitry for male social behavior may be differently involved in the regulation of behaviors (Newman, 1999). Abundant but somewhat differential expression of ERs and ARs in this neural network (Simerly et al., 1990; Shughrue et al., 1997; Mitra et al., 2003; Merchenthaler et al., 2004) indicated that male social behaviors are site-specifically regulated by testosterone in these brain regions via ERs and/or ARs. Among these brain areas, the MPOA and MeA, in which both ERα and ERβ are abundantly expressed, have been focused as regulatory sites of male social behaviors via ERs. Local testosterone implants into the MPOA or MeA restored sexual behavior of castrated male hamsters (Wood and Newman, 1995). Several lines of evidence demonstrated an importance of estrogenic signaling. For instance, Wood (1996) reported that local administration of estradiol but not DHT in the MeA could restore male sexual behavior after in castrated hamsters. Similarly, in the MPOA, local administration of estradiol was more effective than DHT in restoring sexual behavior in castrated male rats (Hull and Rodoriguez-Manzo, 2009).

Sano et al. (2013) reported brain site-specific regulation of male social behaviors.

Effects of site-specific knockdown of ERα (αERKD) in the MPOA, MeA, or VMN on male sexual and aggressive behavior were examined in adult male mice. As a result, αERKD in the MPOA decreased sexual behavior without affecting aggressive behavior, whereas αERKD in the MeA affected neither sexual nor aggressive behavior. On the other hand, αERKD in the VMN reduced both of sexual and aggressive behavior. Recently, Lee et al. (2014) also provided evidence of importance of ERα expressing neurons in the VMN. They reported that activation of ERα positive neuron in the VMN induced social investigation toward the opponent, sexual behavior, and aggressive behavior in scalable manner.

Sano et al. (2016) revealed that ERα might also be site-specifically involved in the formation and/or development of neural networks for male social behaviors in pubertal period. Site-specific αERKD at postnatal day (PND) 21 in the MeA, which continuously suppressed ERα expression from pubertal period to adult, reduced sexual and aggressive behaviors in adulthood. Considering the finding of negative effects of αERKD in the MeA only in adulthood discussed above (Sano et al., 2013), these findings indicate that ERα in the MeA may be necessary for the pubertal organization of neural network for male sexual and aggressive behaviors.

1.7. Possible regulation of male social behavior by ERβ

Compared with ERα, the precise role of ERβ in the regulation of male social behavior still remains unclear. Behavioral alteration by βERKO in male mice has been investigated in previous studies. Ogawa et al. (1999) reported survival of sexual behavior and partially increased aggressive behavior in βERKO male mice. Adult male βERKO mice showed longer duration of and shorter latency to aggressive behavior than wild-type (WT) mice in the first test of three repeated aggressive behavior tests. Increased levels of aggressive

behavior in pubertal and adolescent periods in βERKO males (Nomura et al., 2002) also suggested an inhibitory role of ERβ in the regulation of male aggressive behavior. Moreover, it is hypothesized that ERβ modulates aggressive behavior induced by activation of ERα at an adequate level. Increased level of aggressive behavior induced by estrogen treatment in gonadectomized βERKO suggested potentiation of estrogen-inducible aggression by disruption of ERβ gene (Nomura et al., 2006). Not only the performance of typical aggressive behavior, but also the reaction to social stimuli may be modulated by ERβ. In the situation of encounter to another mouse without direct physical contact, hyper-reactivity has been reported in both male (Handa et al., 2012) and female (Tsuda et al., 2014) βERKO mice.

Moreover, ERβ is implicated in social information processing. In social recognition test using habituation-dishabituation paradigm (see 1.2.1 for details of the paradigm), βERKO female (Choleris et al., 2003) but not male (Sánchez-Andrade and Kendrick, 2011) mice showed disrupted social recognition of same-sex stimulus animals. Although ERβ may play a minor role in social recognition of same-sex conspecifics, ERβ might have a role in recognition of opposite-sex individual in male mice. Kavaliers et al., (2008) revealed that risk-taking behavior was altered in WT, but not in βERKO, male mice by the degree of familiarity of female exposed before the test. Thus, βERKO males possibly are unable to distinguish familiar and novel females.

Additionally, it is known that ERβ possibly mediates anxiolytic effect of estradiol in rodents. Selective ERβ agonist reduced anxiety-related behavior in contrast to anxiogenic effects of ERα agonist in gonadectomized female rats (Lund et al., 2005). Similar behavioral effects of ERβ agonist treatment were reported in gonadectomized males in social and non-social situation (Weiser et al., 2008). It is hypothesized that ERβ may be involved in the maintenance of adequate expression levels of male social behaviors,

which is necessary for animal's survival, not only by direct regulation of stereotypical social behaviors but also by the regulation of emotional aspect.

Previous studies also have suggested ERβ-mediated organizational action. Female-type sexual behavior in hormonally treated βERKO male mice suggests that ERβ may be involved in defeminization of male brains (Kudwa et al., 2005). On the other hand, combined with increased levels of aggression, higher testosterone levels reported in βERKO male at 5 weeks of age (Nomura et al., 2002) suggest that ERβ may play a role in the regulation of puberty onset. Although these studies have not identified the exact period(s) of the organizational action via ERβ, it is possible that ERβ is involved in the formation and development of male-type neural network in neonatal and/or pubertal period.

In a previous study investigating possible neonatal organizational action via ERβ, it is reported that neonatal treatment of male rats with selective ERβ agonist increased aggressive behavior in adulthood (Patisaul and Bateman, 2008). To elucidate complicated role of ERβ in the regulation of male social behaviors, it is necessary to further investigate its organizational and activational action in different stages in lifetime.

1.8. Site-specific knockdown of ERs with RNA interference (RNAi)

1.8.1. Development and mechanisms of RNAi methods

Invention and development of RNAi method enabled us to suppress the expression of a targeted gene. This technique originated from the finding that hairpin-shape short RNA interfered gene expression (Lee et al., 1993). Subsequently, Fire et al. (1998) succeeded to inhibit gene expression by introduction of double-strand RNA into cell. These findings and subsequent development of RNAi methods enabled site- or cell type-specific knockdown of a targeted gene by introduction of small double-strand RNA.

Small hairpin RNAs (shRNA) incorporated to adeno-associated virus (AAV) or lenti virus in plasmid vector are often used for introduction of the RNA. After introduction into the cell, double-strand RNA is converted to siRNA which is single-strand RNA with about 21 base long. Conversion to siRNA enabled introduced RNA to inhibit expression of a targeted RNA (Ghildiyal and Zamore, 2009; Kim et al., 2009; Siomi, H. and Siomi, M.C., 2009). Double-strand RNA is processed by dicer and Argonaute proteins. After the processing, siRNA and Argonaute protein form RISC complex. Targeted RNA with RISC binding is cut by slicer activity of Argonaute protein and decomposed by other RNAase. Thus, this process selectively inhibits translation of the targeted RNA and expression of the targeted gene.

1.8.2. Site-specific knockdown of ERs using RNAi method

AAV vector for brain site-specific knockdown of ERα was constructed by Dr. Sergei Musatov. Musatov et al. (2006) for the first time, succeeded in site-specific knockdown of ERα in the VMN of female mice and provided definitive evidence that ERα in the VMN play an essential role in female sexual behavior. As described above, Sano et al. (2013) investigated the site-specific regulation of male sexual and aggressive behavior by ERα in male mice using the same method. Likewise, Cushing et al. (2008) demonstrated that ERα in the MeA play a role in male prosocial behavior in adult male prairie voles. Figure 3 illustrates the construct of a viral vector for ERα knockdown (shRNA ERα) or a control vector (shRNA LUC) used in these studies. Viral infection induces simultaneous expression of Green Fluorescent Protein (GFP), which enables to visualize injection site and spread of the virus in the targeted brain site. Recently, AAV vector for knockdown of ERβ was constructed by a research team of Dr. Sergei Musatov at the Cornell Medical School and Dr. Sonoko Ogawa at the University of Tsukuba. Construct of the viral vector

for ERβ knockdown is similar to that described in Figure 3.

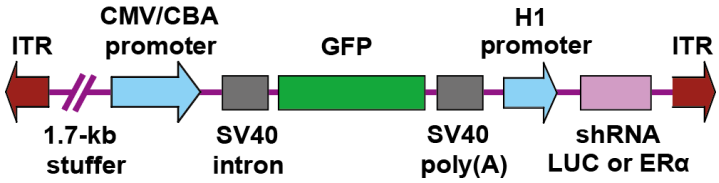

Figure 3. Schematic representation of the AAV vector construct for ERα knockdown or control (modified from Sano et al, 2013)

1.9. Thesis objectives

In this thesis, I aimed to investigate the role of ERβ in the regulation of male social behaviors. Precise role of ERβ in male social behavior is not completely understood. As described above, ERβ may play an important role in modulation of social behaviors, which are possibly turned on by ERα. Investigation of relative importance of ERα vs ERβ, and possible differences in mechanisms of action must contribute greatly to better understanding of precise mechanisms of behavioral regulation by testosterone. Furthermore, ERβ-mediated social information processing may play a role in establishment of social relationship in male mice. However, responsible brain site(s) of ERβ action is virtually unknown. Thus, in this thesis, I aimed to investigate the role of ERβ in social behavior regulation from two aspects. The first question was *how, where and when each component of male social behaviors, such as social information processing, sexual and aggressive behavior, is regulated by ERβ?* Site-specific knockdown using RNAi methods enabled us to investigate site- and age- specific role of ERβ in the regulation of social behaviors in male mice. Secondly, in addition to the regulation of each component of social interaction, *relative importance of ERβ in actual social interaction; choice of an appropriate partner for mating and establishment of*

social relationship to other males was examined.

In experiments 1-4, I examined the effects of pre-pubertal or adult site-specific knockdown ERβ in the MPOA and MeA on male social behaviors. Among several expression sites, the MPOA and MeA express both ERα and ERβ abundantly, and are known to be responsible for male social behaviors (Kondo, 1992; Paredes et al., 1993; Hull et al., 1999, Kondo and Sachs, 2002; Patil and Brid, 2010; Wang et al., 2013). I first tested whether ERβ in the MPOA or the MeA is necessary for male sexual and aggressive behaviors by site-specific knockdown of ERβ (βERKD) before puberty. I then examined the influence of site-specific βERKD only in adulthood in the MPOA. In the MeA, I intended to examine the influence of adult βERKD on social information processing including male-type sexual preference and social recognition in addition to sexual and aggressive behaviors since the MeA is known to play a pivotal role in social information processing (Ferguson et al, 2002; Baum, 2009). I also assessed an effect of βERKD in the MeA on partner choice during actual sexual behavior tests in Experiment 4. By comparing the influence of pre-pubertal and adult βERKD, I aimed to elucidate the roles of pubertal and adult ERβ in the MPOA and MeA.

In Experiment 5, I aimed to investigate whether ERβ plays a significant role not only in the regulation of stereotypical social behaviors in a single encounter, but also repeated social interaction and establishment of social relationship. The role of ERβ in the establishment of hierarchical inter-male social relationship between two males was examined using adult βERKO mice.

Summary of Objective

1) Investigate the effects of pre-pubertal site-specific knockdown ERβ in the MPOA and MeA on social behaviors of male mice.

2) Investigate the effects of adult site-specific knockdown ERβ in the MPOA and MeA on social behaviors of male mice.
3) Investigate the effects of deletion of ERβ gene on establishment of social relationships in male mice.

-Chapter 2-

General Methods

2. General Methods

2.1. Experimental animals

2.1.1. Mice

Gonadally intact ICR/Jcl male mice were used as experimental animals in Experiments 1-4. They were originally purchased from a commercial breeder (CLEA Japan Inc., Japan) and maintained in a breeding colony at the University of Tsukuba. In Experiment 5, βERKO male mice were used. βERKO mice were originally created in C57BL/6J and 129 background by Dr. Kenneth S. Korach's group at the National Institute of Environmental Health Sciences (Krege et al., 1998). Heterozygous breeding pairs completely backcrossed to C57BL/6J were then gifted to Dr. Sonoko Ogawa at the University of Tsukuba. They were also maintained in a breeding colony at the University of Tsukuba. All mice were kept under standard housing conditions (23±2°C, 12:12 light/dark cycle with lights off at 12:00) in polypropylene clear plastic cages (19x29x12 cm; Allentown Inc., USA) with corncob bedding (Greentrue, Purina PetCare Co., USA). Food (Rodents Diet MF, Oriental Yeast Co., Ltd., Japan) and water were provided *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health guidelines and were approved by the Animal Care and Use Committee and the Recombinant DNA Use Committee at the University of Tsukuba. All efforts were made to minimize the number of animals and their suffering.

2.1.2. Tail DNA extraction and PCR genotyping of βERKO mice

To identify genotype of each subject animal, mouse genomic DNA from tail tip samples of βERKO mice (used in Experiment 5) were collected on the day of weaning (PND21). Samples for PCR genotyping were prepared with a Hot Sodium Hydroxide and

Tris (HotSHOT) method (Truett et al., 2000). Tail samples were incubated in an alkaline lysis reagent (25 mM NaOH, 0.2 mM EDTA·2Na; pH 12) for 1hr at 95°C. Thereafter, samples were cooled to 4°C and a neutralizing reagent (40 mM Tris-HCl; pH 5) was then added for pH adjustment. DNA was stored at 4°C until used for PCR amplification.

Genotyping of tail DNA was performed by PCR amplifications of ER gene fragments as previously described (Krege et al., 1998). Intron 2 (5'-TGGACTC-ACCACGTAGGCTC-3'), exon 3 (5'-CATCCTTCACAG GACCAGACAC-3') and the 3' end of Neo (5'-GCAGCCTCTGTTCC ACATACAC-3') primers were used. Each tail DNA sample was blended with the above three primers, a standard PCR cocktail mix (10x PCR buffer and 2mM dNTP), and Taq DNA polymerase. All samples were run in the following PCR conditions: denaturation at 94°C for 30s, annealing at 56°C for 30s, elongation at 72°C for 60 s and each cycle was repeated for a total of 36 cycles. PCR samples were run in a 2% agarose gel at 130 V for 30 min. A 1,435bp band (intron 2 and exon 3 primers) is amplified for homozygous wild-type (+/+) mice, 1,479bp band (intron 2 and Neo primers) for homozygous mutant (-/-) mice, and both bands for heterozygous (+/-) mice. In Experiment 5, homozygous wild-type (WT) and homozygous mutant (βERKO) animals were used as experimental mice.

2.2. Estrogen receptor β silencing using small hairpin RNA

In Experiments 1-4, experimental animals were stereotaxically injected with shRNA expressing AAV vectors either on PND 21 (Experiment 1) or in adulthood (Experiments 2-4). AAV-shRNA against the sequence specific for the ERβ gene (AAV-shERβ: 5′-GATCCCCGCCACGAATCAGTGTACCATCTTCCTGTCAATGGT ACACTGATT CGTGGCTTTTTTGGAAT-3′ and 5′-CTAGAGCCACGAATCAGTG TACCATTGACAGGAAGATGGTACACTGATTCGTGGCGGG-3′) was used. AAV-

shRNA against the sequence specific for luciferase (LUC) (AAV-shLUC: 5′-GATCCCCCCGCTGGAGA GCAACTGCATCTTCCTGTCAATGCAGTTGCTCT CCAGCGGTTTTTGGAA-3′ and 5′-CTAGTTCCAAAAACCGCTGGAGAGCAACTGCATGAGCAACTGCATTG ACAGGAAGATGCAGTTGCTCTCCAGCGGGGG-3′) was also used as control. The nucleotides specific for ERβ and LUC are underlined. These vectors also express enhanced GFP as a reporter to visually detect transfected cells.

Mice were anesthetized with sodium pentobarbital (60mg/kg; Kyouritsu Seiyaku Co. Ltd., Japan) and placed in a stereotaxic frame (Model 900, David Kopf Instruments, USA). A 26G injection needle attached to a 10 μl Hamilton syringe was inserted by aiming either at the MeA or MPOA (coordinates were determined for each experiment separately). Each animal was bilaterally injected with 1 μl of either AAV-shERβ or AAV-shLUC (10^{12} packaged genomic particles, 0.5 μl/hemisphere) over 5 min. The needle was left in place for an additional 10 min following the end of the infusion.

2.3. Behavioral tests

All mice were individually housed in the plastic cages starting at least 7 days before the first behavior test. Time course of behavioral assay in each experiment is described in each chapter.

2.3.1. Sexual behavior test

Each experimental animal was tested for sexual behavior against a receptive female mouse in its home cage. Each trial was 30 min and conducted under red light illumination during the dark phase of the light/dark cycle. At the beginning of each trial, a hormonally primed ovariectomized (OVX) ICR/Jcl female stimulus mouse was introduced. All

stimulus animals were obtained from the breeding colony maintained at the University of Tsukuba. To ensure high sexual receptivity, all females were subcutaneously (*s.c.*) injected with 10 μg estradiol benzoate (EB) in 0.1 ml sesame oil at 48 and 24 h and 500μg progesterone (P) in 0.1 ml sesame oil at 4-6 h before testing. Each male was tested against a different female mouse in each of the repeated trials. The cumulative number of mounts and intromissions, and the latency to the first mount or intromission were recorded.

2.3.2. Aggressive behavior test

Aggressive behavior was assessed in a resident-intruder paradigm for 15 min under red light illumination during the dark phase of the light/dark cycle. One test consisted of three trials conducted in three consecutive days. At the beginning of the test, an age-matched gonadally intact ICR/Jcl male mouse (intruder) was introduced into a home cage of an experimental animal (resident). All intruder mice were olfactory bulbectomized and group-housed (3-5 animals per cage). OBX was conducted to inhibit offensive aggression by intruders. Each resident mouse was tested against a different intruder mouse in each of the repeated aggression tests. An aggressive bout was defined as a series of behavioral interactions consisting of at least one of the following: chasing, boxing, tail rattling, wrestling, biting, and offensive lateral attack (often accompanied by biting). The cumulative number and duration of aggressive bouts were recorded. A maximum of three seconds could elapse between two aggressive bouts to be considered as one aggressive bout. If the interval exceeded three seconds, the two bouts were scored as two separate aggressive bouts.

2.3.3. Sexual preference tests

In sexual preference tests, preference toward two different stimulus mice was tested.

In olfactory sexual preference test (2.3.3.1.), experimental animals were prevented from direct interaction with stimulus animals. In two-female sexual behavior test (2.3.3.2.), males were allowed direct physical contact with females.

The testing apparatus consisted of a white plastic testing cage (31x35x17 cm) placed centrally in a white polyvinyl chloride box (46x51x25 cm). Testing cage was covered with a clear acrylic board during tests and a video camera was placed 57 cm from the bottom of the testing cage.

2.3.3.1. Olfactory sexual preference test

In Experiments 2 and 3, each experimental mouse was tested for sexual preference of a receptive female over a non-receptive female (PTFF) and a receptive female over an intact male (PTFM). In Experiment 3, each experimental mouse was tested for preference of a gonadectomized male over intact male (PTMM) in addition to PTFF and PTFM. In PTFF, a hormonally primed (see 2.3.1.) OVX C57BL/6J female mouse (receptive female: RF) and an OVX C57BL/6J female without hormonal priming (non-receptive female: XF) were used as stimulus animals. In PTFM, a RF and a gonadally intact C57BL/6J male (IM) mouse were used. In PTMM, a gonadectomized C57BL/6J male (XM) mouse and an IM were used. Each test was 15 min and conducted under white light illumination (26 lux) during the dark phase of the light/dark cycle. Clear sectoral Plexiglas cylinders (7 cm in radius, 16 cm in height) with 13 holes (6 mm diameter) near the bottom 3 cm (Mouse Cylinder SIOT3, O'Hara & Co., Ltd., Japan) were used to present stimulus mice. Experimental mice were able to sniff olfactory cues from stimulus mice through perforated parts of the cylinders.

At least two days before testing, each experimental mouse was transferred to a testing cage with clean bedding and allowed to establish its own home territory. On the day of

the testing, they were first habituated to two empty cylinders for one hour. The cylinders were placed at diagonal corners of the testing cage. At the beginning of the test, empty cylinders were removed and two cylinders with stimulus animals were placed at the same two diagonal corners. After completion of each test, cylinders were thoroughly washed, wiped with 70% ethanol, and then air-dried.

Social investigation (SI) was defined as sniffing toward each stimulus animal through the holes of the cylinder (Figure 4). The cumulative duration of SI to each stimulus mouse was recorded separately. A maximum of one second could elapse between two SIs to be considered as one bout. If the interval exceeded one second, they were recorded as two bouts.

Figure 4. Social investigation of an experimental mouse (white mouse) in olfactory sexual preference test.

2.3.3.2. Sexual preference test with freely moving two females (2F Sex test)

At least one week before testing, subject mice was transferred to a testing cage and allowed to establish home territory. Each trial was 30 min and conducted under red light illumination during the dark phase of the light/dark cycle. At the beginning of the test, two ovariectomized ICR/Jcl female stimulus mice were introduced into subject's cage. One of the females was hormonally primed as described in 2.3.1. to ensure high sexual

receptivity (RF). On the other hand, another female was not hormonally primed (XF). In this test, latency to first mount and intromission to each stimulus mouse was recorded separately to evaluate which stimulus female was chosen as a partner for sexual behavior.

2.3.4. Social recognition test

Each experimental mouse was tested for social recognition with RF, XF, and IM mice. Each test was conducted under white light illumination (26 lux) during the dark phase of the light/dark cycle. Test apparatus other than the cylinder was same as sexual preference tests. One empty round cylinder (7 cm in diameter at the bottom and 4.4 cm in diameter at the top, 16cm in height) with 28 holes (6 mm diameter) near the bottom 3cm (Tsuda and Ogawa, 2012; Mouse Cylinder SIOT1, O'Hara & Co., Ltd.) was introduced in the center of testing cages 1 h before the first trial. Experimental mice were tested four times, 4 min each, with 17 min inter-trial intervals (Figure 5). In the first three trials, each experimental mouse was tested against the same stimulus mouse (Stimulus A) whereas in the fourth trial, he was tested against a different (novel) stimulus mouse (Stimulus B). Same types of mice (*i.e.,* RF, XF or IM) were used for Stimuli A and B. The cumulative duration of SI was recorded in each trial. Definition of SI was the same as that in olfactory sexual preference test (see 2.3.3.1.).

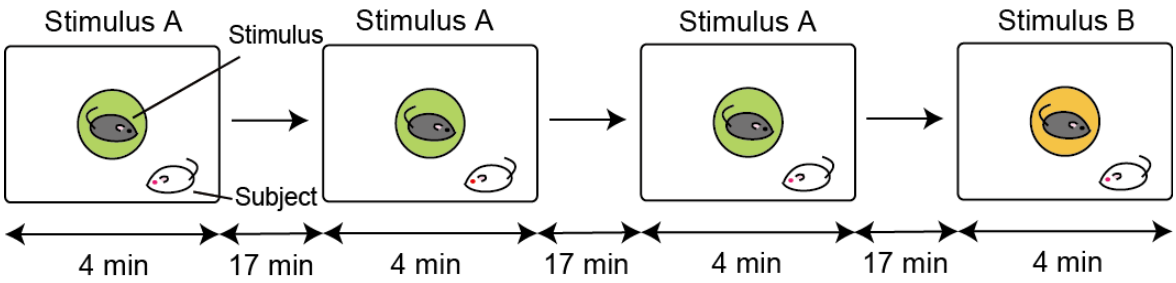

Figure 5. Schema of experimental procedure for social recognition test.

2.3.5. Agonistic behavior test

Agonistic behavior between two experimental male mice was assessed in a neutral testing cage (a plastic cage of the same type as animals' home cage) for 15 min under red light illumination during the dark of the light/dark cycle. Before each testing trial, testing cage was separated into two compartments by a black Plexiglas board (divider). Each experimental animal was placed in each compartment and was habituated to the testing environment for 5 min. At the beginning of the test, the divider was removed and agonistic behaviors were observed. Number and duration of following behaviors were recorded. The cumulative number and duration of aggressive behavior (definition is described in 2.3.2.), fleeing, approaching, sniffing, huddling, and grooming, and the cumulative number of tail rattling were recorded. These behavioral indices were classified into either agonistic or prosocial interaction. Agonistic interaction was defined as a series of behavioral interactions consisting of at least one of the following: aggressive behavior, fleeing, and tail rattling. For calculation of the cumulative duration of agonistic interaction, cumulative duration of aggressive behavior and fleeing were added. Prosocial interaction was defined as a series of behavioral interactions consisting of at least one of the following: approach, sniffing, huddling and grooming.

2.3.6. Tube test

Tube test was conducted using testing arena (70x50 cm) surrounded by black wall. A transparent Plexiglas tube (length: 45 cm, inner diameter: 3 cm) was set on the center of the testing arena.

2.3.6.1. Training

In each training trial, each experimental animal was forced to run through the tube

from one end to the other end. A black plastic escape box (13x14x13 cm) was attached at the end of the tube in some of the training trials. Each experimental animal experienced 8 trials per day for two consecutive days. On the first day of the training, mice experienced initial 4 training trials with the escape box and 4 trials thereafter without the escape box. On the second day, initial 2 trials were conducted with the escape box and the rest of the trials were without the escape box. At the begging, each animal was gently held and put into one end of the tube. Starting side in initial trial was counterbalanced. When a mouse stopped in the tube, an experimenter gently pushed animal's back with a plastic pole. After a mouse reached the end, he was trained to run in an opposite direction. At the end of training of each animal, all apparatuses were wiped with 70% ethanol and air-dried.

2.3.6.2. Testing

Before the testing trial on each day, two training trials without escape box were conducted. At the begging of the testing trial, a pair of experimental animals was set on each end of the tube and an experimenter released the mice to let them run into the tube (Figure 6, left). Starting side of each animal was counterbalanced. The test trial ended when one of the mice was ejected from the end where he first entered (Figure 6, right). A mouse stayed inside of the tube at the end of the trial was called as a "winner" and an ejected mouse was called as a "loser". Alternatively, if two minutes elapsed without ejection of either mouse, the trial ended as a "tie". At the end of each test trial, all apparatuses were wiped with 70% ethanol and air-dried.

Winner's animal ID and the latency to the end of trial were recorded in each trial. Furthermore, occurrence of "invasion" by a winner was recorded. When both hind paws of a winner crossed mid-point of the tube to loser's side, invasion was recorded (Figure 6, right). The loser was able to walk back spontaneously and exit the tube even if it was

not pushed by the winner.

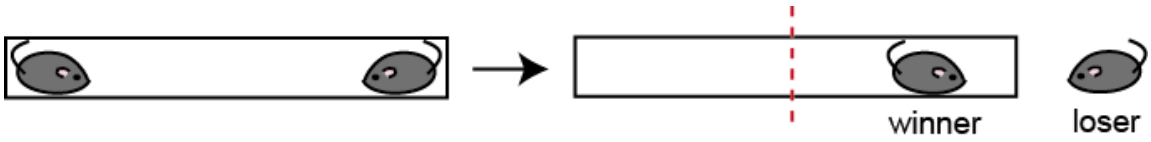

Figure 6. Schema of experimental procedure for tube test. At the beginning of the test, mice were released and run into the tube (left panel). The test ended after ejection of a loser (right panel). Invasion: both hind paws of a winner crossed mid-point of the tube (red line of the right panel) to loser's side.

2.3.7. Quantitative analysis of behavioral data

All behavioral tests were recorded using digital video cameras. All video recordings were scored by an experimenter unaware of animals' experimental group using a digital event recorder program (Recordia 1.0b, O'Hara & Co., Ltd.).

Behavioral data from sexual and aggressive behavior tests was analyzed by a two-way analysis of variance (ANOVA) for repeated measurements for the main effects of vector treatment, tests and their interactions. The data from sexual preference tests except for total SI duration in olfactory sexual preference test was analyzed in each vector treatment group separately by a paired t-test between two stimulus mice. Total SI duration in olfactory sexual preference test was analyzed by an unpaired t-test between vector treatment groups. Average SI duration in social investigation test was analyzed in each vector treatment group separately by one-way ANOVA for repeated measurements for three types of stimulus mouse. Behavioral data from agonistic behavior tests and tube test was analyzed by a two-way ANOVA for repeated measurements for the main effects of genotype, days, and their interaction. Post hoc analysis was conducted with Bonferroni correction when interaction was significant. All these data were analyzed using the SPSS ver. 21.0 (SPSS Inc., USA). Proportion difference in the test trials in Experiment 5 was

analyzed in Fischer's Exact Test. Fischer's Exact Test was conducted using the R (The R Project for Statistical Computing). Statistically significant differences were considered when $p<0.05$.

2.4. Histological analysis

In Experiments 1-4, histological analysis was conducted after behavioral tests.

2.4.1. Preparation of brain tissues for immunohistochemistry

After the completion of the last behavioral tests, all experimental animals were deeply anesthetized with heparin-containing pentobarbital sodium solution (60 mg/kg body weight, *i.p.*). They were then perfused through the left cardiac ventricle with 40 ml of 100 mM phosphate buffered saline (PBS; pH 7.2) for blood removal, followed by 40 ml of 4% paraformaldehyde-containing 100mM phosphate buffer (PB; pH 7.2) for fixation with the use of a peristaltic pump. Brains were removed and post-fixed in the same fixative at 4℃ for 24h. After cryoprotection in 30% sucrose in 100 mM PB at 4℃, coronal sections (30 μm thickness) were prepared using a freezing microtome. Serial sections were collected in four sets with 120 μm intervals, and stored in anti-freezing buffer (30% ethylene glycol and 30% glycerol in 0.05 M Tris-buffered saline (TBS), pH 7.2) at -20°C until use.

2.4.2. Immunohistochemistry

Freely floating sections were incubated in PBS containing 0.2% triton X (PBS-X) with 0.3% H_2O_2 for 20 min at room temperature (RT) for blocking. After washing, sections were pretreated with 5% bovine serum albumin (BSA) in PBS–X (blocking buffer) for 2 h at RT. The sections were then incubated with goat polyclonal anti-GFP

antiserum (1:5,000; ab6673, Abcam, USA) in blocking buffer for one night at 4℃. They were washed and incubated with biotinylated rabbit anti-goat secondary antiserum (1:250; Vector Laboratories) in blocking buffer for 2 h at RT. After washing, sections were reacted to avidin-biotin complex (Vectastain ABC Elite kit; Vector Laboratories) PBS for 1 h at RT, and washed. They were then incubated in 0.02% (DAB) and 0.003% H_2O_2 in PBS for 2 min, followed by wash with PBS. A few sections from each group were also processed for double immunohistochemical staining for GFP and ERβ. Prior to immunohistochemistry for GFP, they were incubated with rabbit polyclonal ERβ antiserum (1:1000; Z8P, lot 10766190, Zymed Laboratories, USA) for 3 days at 4°C followed by biotinylated goat anti-rabbit secondary antiserum (1:250; Vector Laboratories) for 2 h and visualized in 0.03% diaminobenzidine (DAB), 0.15% NiNH4SO4, and 0.003% H_2O_2 in TBS for 12-14 min, followed by wash with TBS (pH 7.2).

All sections were mounted on gelatin-coated slides, air-dried, dehydrated through ascending series of ethanol, cleaned with xylene, and coverslipped with Permount (Fisher Scientific, USA).

2.4.3. Analysis of immunopositive cells

Nine sections containing the MPOA (Bregma 0.38 to -0.58) and nine sections containing the MeA (Bregma -1.10 to -2.06) were selected for histological analysis of immunopositive cells for GFP. Each brain area was photographed at 20x magnification with a digital camera mounted on a microscope (BZ-X710, KEYENCE Corporation, Japan). Spread of GFP immunopositive cells were recorded for confirmation of AAV infection in the targeted area. We also selected three double-immunostained sections in the MPOA (Bregma 0.02, -0.10, and -0.22) and in the MeA (Bregma -1.82, -1.94, and -

2.06) where most intensive ERβ expression was observed in the control groups. In these sections, we counted (3 mice per group) number of ERβ-immunopositive cells and double-labeled cells for ERβ and GFP in each side of the hemisphere within the targeted site. The data was analyzed in each section separately by a Welch's t-test between two vector treatment groups using the SPSS ver. 21.0 (SPSS Inc., USA). Statistically significant differences were considered at $p < 0.05$.

-Chapter 3-

Experiment 1:

Effects of Pre-Pubertal ERβ Knockdown in the MPOA and MeA

3. Experiment 1: Effects of Pre-Pubertal ERβ Knockdown in the MPOA and MeA

3.1. Introduction

It is still unknown when, where in the brain, and how testosterone regulates male social behavior via ERβ. In addition to androgen surge in perinatal period, circulating testosterone level starts to increase from the beginning of pubertal period and reaches to adult level at the end of puberty. Thereafter, activation of adult neural network, which is formed and developed by perinatal and pubertal organizational action of testosterone, induces a variety of male social behaviors.

Although it is known that ERβ is involved in the regulation of male social behavior (Ogawa et al., 1999; Nomura et al., 2002, 2006; Kavaliers et al., 2008), precise time course of its action is still unclear. Previous study using selective ERβ agonist has reported that ERβ activation in the perinatal period can facilitate aggressive behavior in adulthood (Patisaul and Bateman, 2008). Thus, it is possible that ERβ mediates not only activational, but also organizational action of testosterone. However, the role of ERβ in pubertal period and adulthood in different brain sites remains to be elucidated.

To investigate relative importance of ERβ in pubertal period and adulthood, effects of pre-pubertal site-specific knockdown ERβ in the MPOA and MeA on the performance of sexual and aggressive behavior in adulthood was examined. It is well documented that both MPOA and MeA, play an important role in the regulation of sexual and aggressive behavior (Paredes et al., 1993; Hull et al., 1999; Patil and Brid, 2010). Since knockdown of ERβ in pre-pubertal period suppresses ERβ gene expression permanently after AAV injection, it can be tested whether pubertal and adult ERβ in the MPOA and MeA is necessary for the performance of sexual and aggressive behavior. Moreover, the MPOA

and MeA express high levels of both ERα and ERβ (Shughrue et al., 1997; Mitra et al., 2003). It is intriguing to clarify whether ERβ in these target sites have similar roles as ERα reported by Sano et al. (2013, 2016).

3.2. Methods

A total of 12 litters of ICR/Jcl male mice were assigned to either MPOA or MeA groups on PND 21 after being weaned. Mice from each litter were further divided into two shRNA injection groups of either AAV-shERβ or AAV-shLUC. Those four groups were designated as pre-pubertal treatment (PP)-MPOA-βERKD (n=11), PP-MPOA-Cont (n=13), PP-MeA-βERKD (n=9), and PP-MeA-Cont (n=9). Coordinates for the MPOA group were AP +0.02, ML ±0.5, DV-5.2, and those for the MeA group were AP -1.25, ML ±2.2, DV -5.15. All coordinates were determined based on The Mouse Brain Stereotaxic Coordinates (Paxinos and Franklin, 2001) with an adjustment for the brain size on PND 21. All mice were then group housed with their littermates (4~5 mice per cage) until they were tested for sexual and aggressive behavior in adult as gonadally intact (11.9±0.21 wks old at the first behavioral test). Starting one week before the first behavioral test, all mice were individually housed. Three sexual behavior tests (SEX) and three sets of aggressive behavior tests (AGG) were done in alternate weeks for a total of six weeks (Figure 7). After the completion of the last behavioral test, brain tissues were collected and processed for immunohistochemistry for GFP and ERβ.

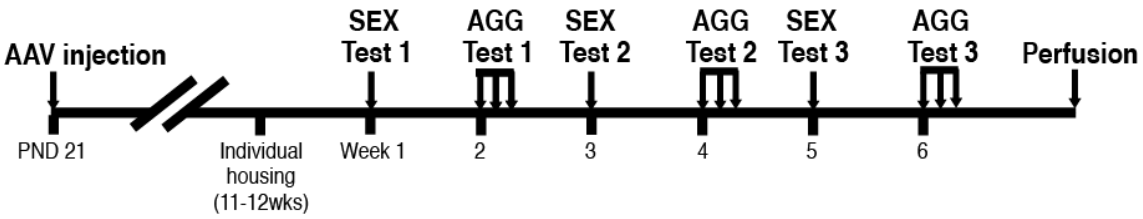

Figure 7. Schema of experimental procedures. Tick marks under the horizontal bar indicate one week. SEX, sexual behavior; AGG, aggressive behavior.

3.3. Results

3.3.1 Effects of pre-pubertal ERβ knockdown in the MPOA

There was no difference in male sexual behaviors between the PP-MPOA-βERKD and PP-MPOA-Cont groups in sexual behavior tests (Figure 8). Statistical analysis revealed that there was no significant main effects of treatment and test, and interaction of treatment and test in any of number of mounts (treatment: $F_{1,18} = 1.117$, *n.s.*; test: $F_{2,36} = 2.631$, $p = 0.086$; treatment x test: $F_{2,36} = 1.770$, *n.s.*) and intromissions (treatment: $F_{1,18} = 0.396$, *n.s.*; test: $F_{2,36} = 0.030$, *n.s.*; treatment x test: $F_{2,36} = 0.302$, *n.s.*), and latency to the first mount (treatment: $F_{1,18} = 0.860$, *n.s.*; test: $F_{2,36} = 0.078$, *n.s.*; treatment x test: $F_{2,36} = 2.161$, *n.s.*). These results indicated that pre-pubertal ERβ knockdown in the MPOA has minimal effects on sexual behavior of adult male mice.

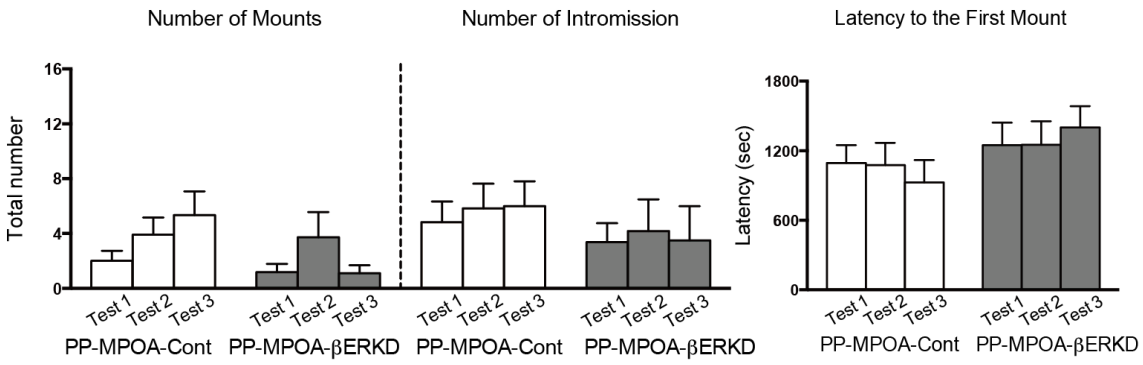

Figure 8. Effect of pre-pubertal βERKD in the MPOA on sexual behavior in adulthood. There was no difference between the PP-MPOA-Cont and PP-MPOA-βERKD groups in either number of mounts (left panel), intromissions (middle panel), or latency to the first mount (right panel). All data are presented as mean+Standard Error of the Mean (SEM).

On the other hand, pre-pubertal ERβ knockdown in the MPOA significantly decreased aggressive behaviors (Figure 9). Statistical analysis revealed that the PP-MPOA-βERKD group showed significantly fewer number (treatment: $F_{1,22} = 4.631$, $p < 0.05$; test: $F_{2,44} = 2.202$, *n.s.*; treatment x test: $F_{2,44} = 0.851$, *n.s.*) and shorter duration (treatment: $F_{1,22} = 5.078$, $p < 0.05$; test: $F_{2,44} = 0.616$, *n.s.*; treatment x test: $F_{2,44} = 1.654$, *n.s.*) of aggressive bouts compared to the PP-MPOA-Cont group. These results indicated that pre-pubertal knockdown of ERβ inhibited full expression of aggressive behavior in adulthood.

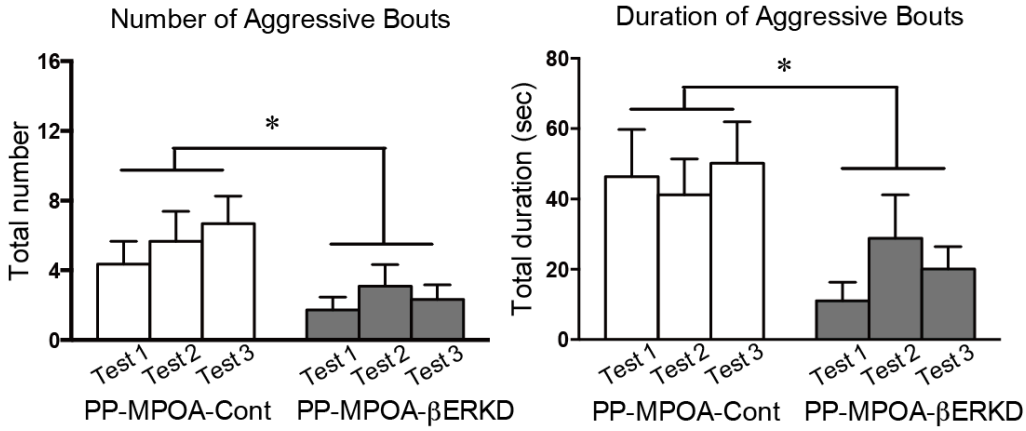

Figure 9. Effect of pre-pubertal βERKD in the MPOA on aggressive behavior in adulthood. Duration (left panel) and number (right panel) of aggressive bouts was significantly reduced in the PP-MPOA-βERKD group compared with the PP-MPOA-Cont group (*$p < 0.05$). Behavioral data are presented as mean+SEM.

3.3.2. Effects of pre-pubertal ERβ knockdown in the MeA

The PP-MeA-βERKD and PP-MeA-Cont groups showed equivalent levels of sexual behavior (Figure 10). Statistical analysis revealed that there was no significant main effect of treatment and test, and interaction of treatment and test in any of number of mounts (treatment: $F_{1,15} = 0.181$, *n.s.*; test: $F_{1.382,20.733} = 2.751$, *n.s.*; treatment x test: $F_{1.382,20.733} = 0.034$, *n.s.*; adjusted by Greenhouse-Geisser) and intromissions (treatment: $F_{1,15} = 1.232$, *n.s.*; test: $F_{2,30} = 1.873$, *n.s.*; treatment x test: $F_{2,30} = 2.927$, $p = 0.069$), and latency to the first mount (treatment: $F_{1,15} = 0.001$, *n.s.*; test: $F_{1.340,20.101} = 0.904$, *n.s.*; treatment x test: $F_{1.340,20.101} = 0.390$, *n.s.*; adjusted by Greenhouse-Geisser).

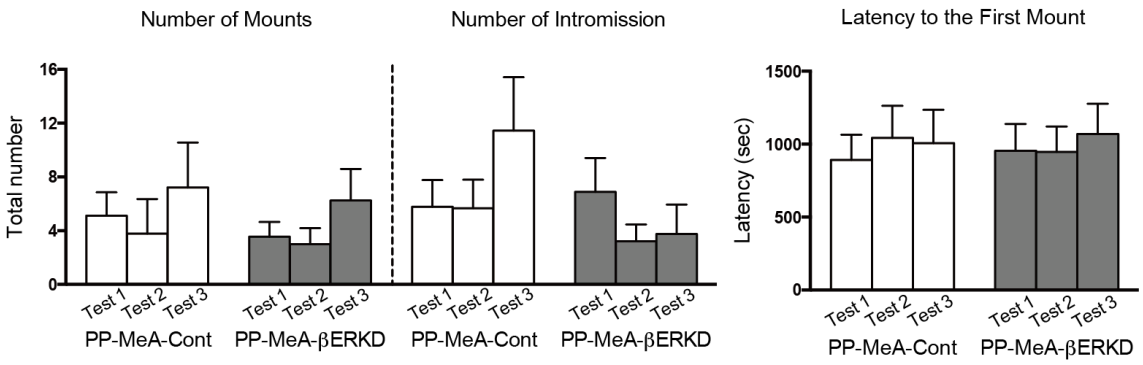

Figure 10. Effect of pre-pubertal βERKD in the MeA on sexual behavior in adulthood. There was no difference between the PP-MeA-Cont and PP-MeA-βERKD groups in either number of mounts (left panel), intromissions (middle panel), or latency to first mount (right panel). All data are presented as mean+SEM.

In aggressive behavior tests, the PP-MeA-βERKD and PP-MeA-Cont groups also showed the same level of aggression throughout three tests (Figure 11). Statistical analysis revealed that there was significant main effect of treatment and test, and interaction of treatment and test in neither of number (treatment: $F_{1,16} = 0.051$, *n.s.*; test: $F_{2,32} = 1.467$, *n.s.*; treatment x test: $F_{2,44} = 0.054$, *n.s.*) nor duration (treatment: $F_{1,16} = 0.232$, *n.s.*; test: $F_{2,32} = 0.206$, *n.s.*; treatment x test: $F_{2,44} = 0.572$, *n.s.*) of aggressive bouts. These results indicated that pre-pubertal ERβ knockdown in the MeA did not affect sexual and aggressive behavior in adult.

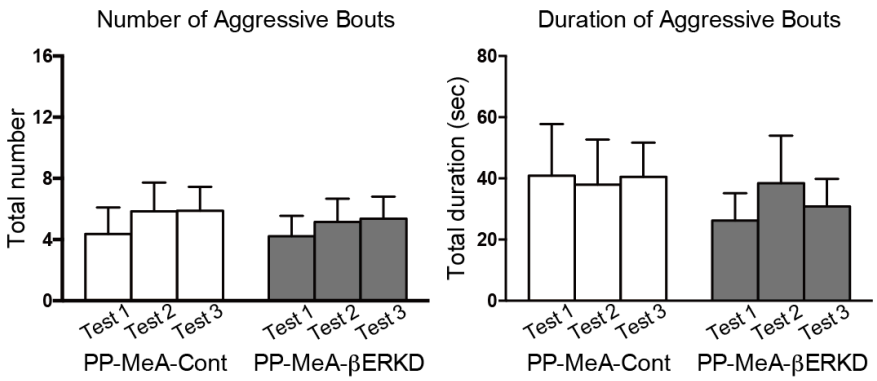

Figure 11. Effect of pre-pubertal βERKD in the MeA on male aggressive behavior in adulthood. There was no difference between the PP-MeA-Cont and PP-MeA-βERKD groups in either duration (left panel) or number (right panel) of aggressive bouts. All data are presented as mean+SEM.

3.3.3. Examination of successful knockdown of ERβ within the targeted brain site

Examination of placement of the injection needle tip (Figure 12A: MPOA; B: MeA) and presence of GFP-immunopositive cells confirmed successful bilateral injections of AAV vectors within the MPOA (Figure 13A) and MeA (Figure 13B) for all mice used in behavioral analysis. In addition, ERβ expression was examined immunohistochemically (n=3/group). The number of ERβ-immunoreactive cells in each targeted site was significantly reduced in the βERKD groups compared with those in the Cont groups (MPOA: Bregma +0.02, $t_{(6.789)} = 2.449$; $p < 0.05$, Bregma -0.10, $t_{(5.147)} = 4.315$; $p < 0.01$, Bregma -0.22, $t_{(5.672)} = 4.171$; $p < 0.01$, Figure 14A; MeA: Bregma -1.82, $t_{(5.739)} = 9.443$; $p < 0.01$, Bregma -1.94, $t_{(7.485)} = 5.267$; $p < 0.01$, Bregma -2.06, $t_{(8.407)} = 9.314$; $p < 0.01$, Figure 14B; Table 1). Furthermore, co-expression of ERβ in GFP-immunopositive cells was detected by double-labeled immunohistochemistry in AAV-shLUC-injected control mice. On the other hand, in AAV-shERβ-injected mice, ERβ expression was absent in the GFP-immunopositive cells, although I found ERβ expression in a few GFP-negative cells

in these mice (Figure 14, bottom panels; Table 1). These anatomical analyses confirmed successful knockdown of ERβ expression in transfected cells in the MPOA- and MeA-βERKD groups.

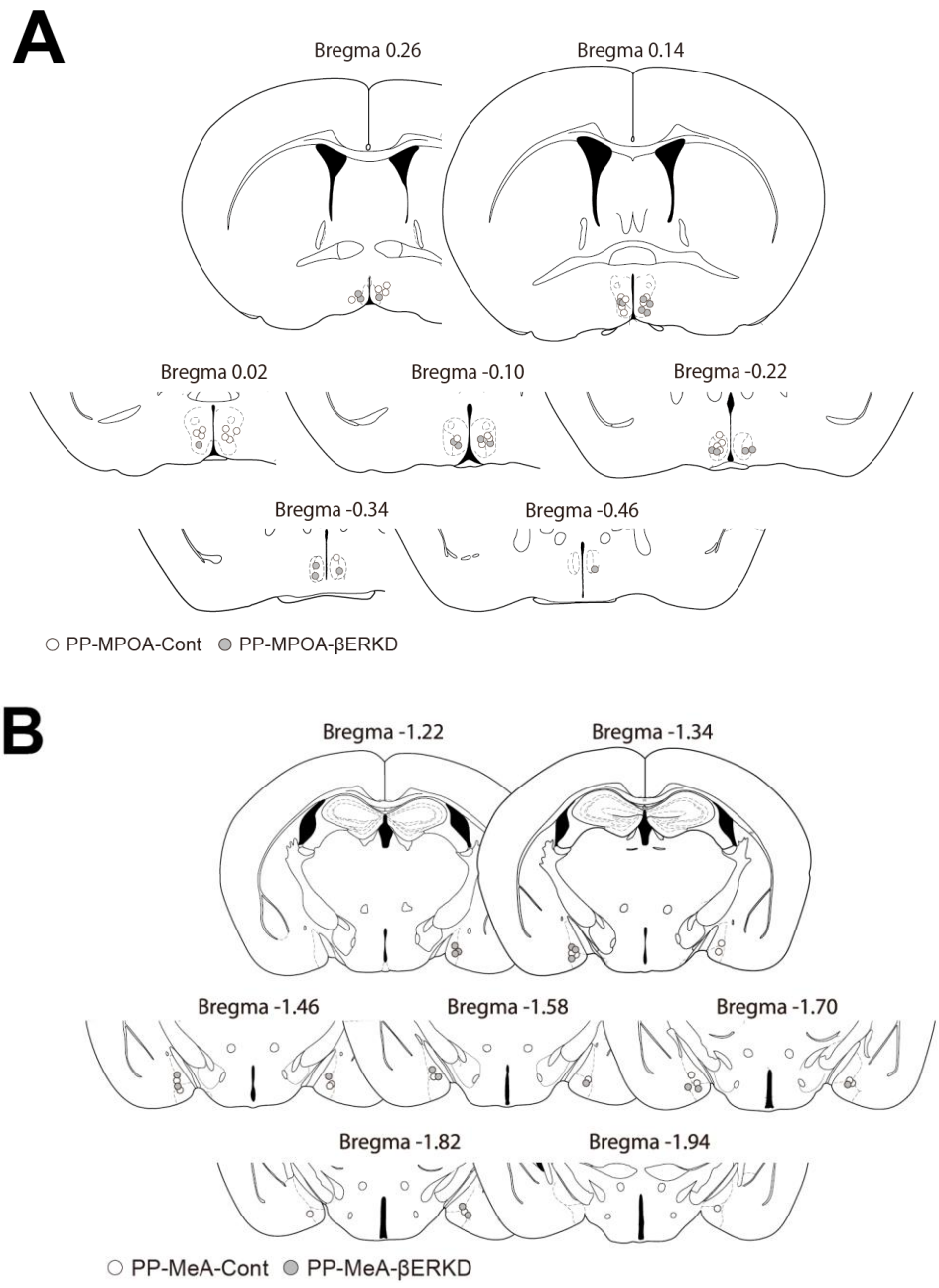

Figure 12. Histological diagrams depicting the placement of the injection needle tip for

each mouse (A) in the PP-MPOA-Cont (open circles) and PP-MPOA-βERKD (solid circles) groups and (B) in the PP-MeA-Cont (open circles) and PP-MeA-βERKD (solid circles) groups.

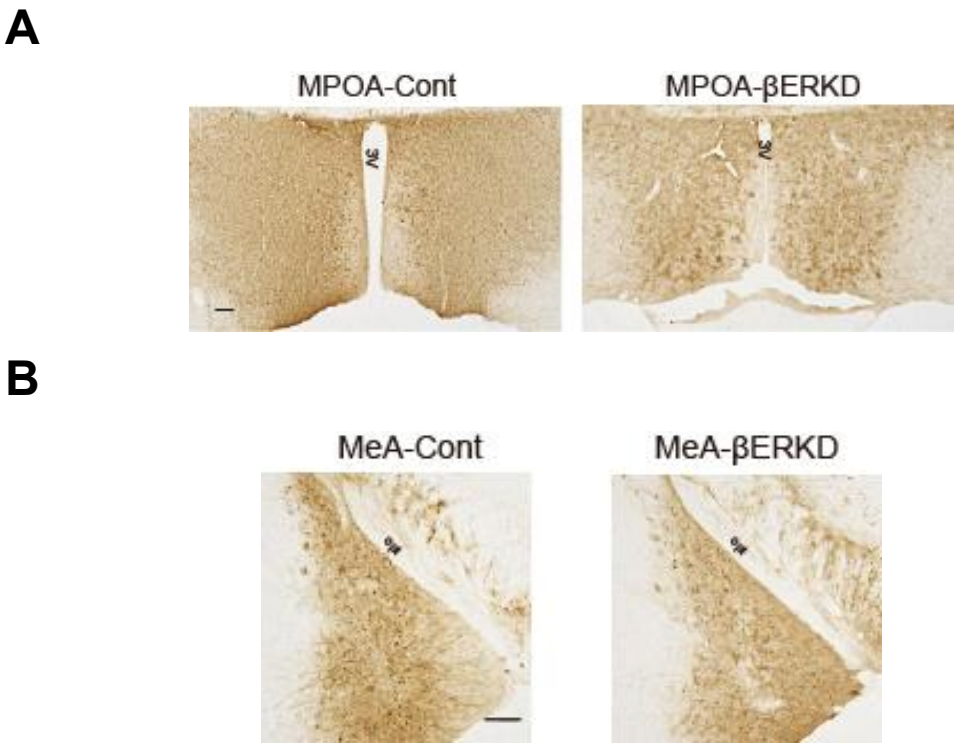

Figure 13. Representative photomicrographs of (A) MPOA sections from PP-MPOA-Cont and PP-MPOA-βERKD mice with single-immunohistochemical staining for GFP (at Bregma -0.10). Scale bar, 100 μm. 3V, third ventricle. (B) MeA sections from PP-MeA-Cont and PP-MeA-βERKD mice with single immunohistochemical staining for GFP (at bregma -1.82). Scale bar, 200 μm. opt, optic tract.

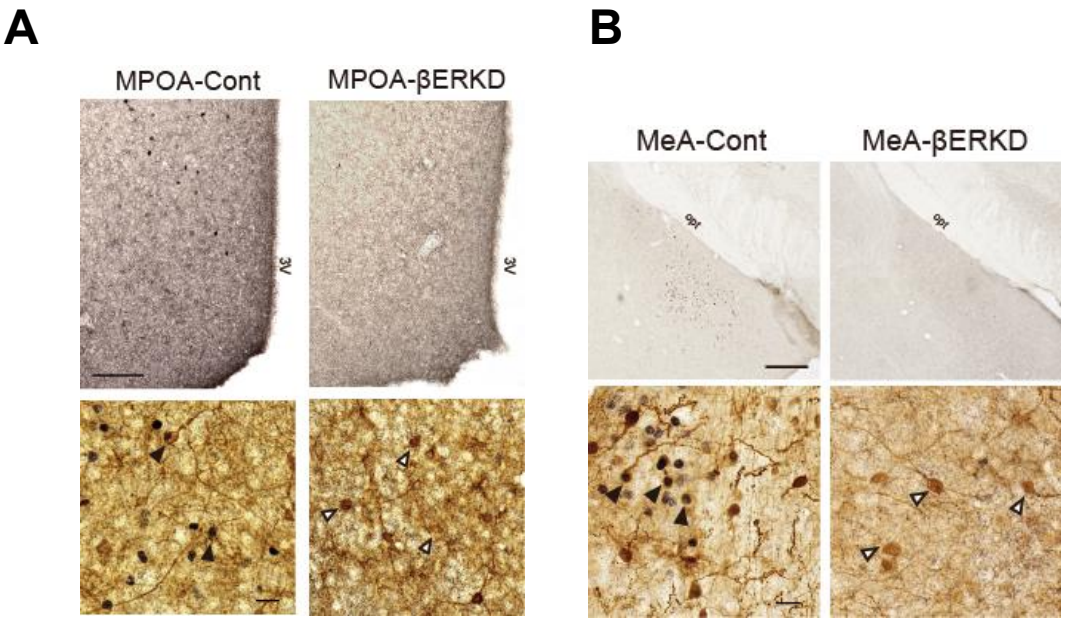

Figure 14. Representative photomicrographs of (A) MPOA sections with single-immunohistochemical staining for ERβ (top; at Bregma -0.22), and MPOA sections with double-immunostaining for GFP and ERβ (bottom). Number of ERβ-immunoreactive cells in the targeted site was reduced in the βERKD group compared with the control group. Scale bars: top, 100 μm; bottom, 20 μm. (B) MeA sections with single-immunohistochemical staining for ERβ (top; at Bregma -1.94), and MeA sections with double-immunostaining for GFP and ERβ (bottom). Number of ERβ-immunoreactive cells in the targeted site was greatly reduced in the βERKD group compared with the control group. Scale bars: top, 200 μm; bottom, 20 μm. (A) and (B), Bottom, Black arrowheads indicate ERβ and GFP double-immunoreactive cells and white arrowheads indicate immunoreactive cells only for GFP.

Table 2. Number of ERβ immunopositive cells

		Control			βERKD		
		Total ERβ	Double-labeled		Total ERβ	Double-labeled	
	Bregma	cell number	cell number	% in total	cell number	cell number	% in total
MPOA	+0.02	20.5±4.7	15.3±3.6	76.2±6.3	8.0±2.0 *	0	0
	-0.10	40.8±8.2	32.0±6.3	79.5±3.8	5.0±1.0 **	0	0
	-0.22	9.8±2.1	7.7±2.1	74.2±5.9	0.8±0.5 **	0	0
MeA	-1.82	158.8±14.7	117.0±9.0	73.9±1.3	15.3±4.0 **	0.7±0.3	3.6±1.8
	-1.94	223.0±19.1	141.8±16.7	63.2±4.8	80.8±19.1 **	1.3±1.3	1.2±1.2
	-2.06	198.7±18.4	210.0±15.5	70.1±1.4	96.3±11.5 **	0.7±0.3	0.8±0.4

Total ERβ cell number, number of ERβ immunopositive cells/side in the targeted area; Double-labeled cell number, number of double-stained cells with ERβ and GFP; Double-labeled % in total, percentage of double-stained cells in the total number of ERβ immunopositive cells. MPOA, medial preoptic area; MeA, medial amygdala; *$p < 0.05$, **$p < 0.01$ vs Control.

3.4. Discussion

In this experiment, site-specific βERKD in the MPOA or MeA from pre-pubertal period to adulthood was conducted. As a result, pre-pubertal βERKD in the MPOA decreased aggressive behavior in adulthood without affecting sexual behavior. Significant reduction, but not abolishment of aggressive bouts in PP-MPOA-βERKD mice was consistent with modulatory role of ERβ which has been suggested in previous studies (Ogawa et al., 1999; Nomura et al., 2002; Handa et al., 2012). These results indicated that ERβ in the MPOA plays facilitatory role in male aggressive behavior.

However, pre-pubertal βERKD in the MPOA didn't affect sexual behavior although the MPOA is highly implicated in the regulation of male sexual behavior (Kondo, 1992, Hurtazo and Paredes, 2005). These results contrast markedly with the result of site-specific knockdown of ERα in the MPOA. Pre-pubertal and adult αERKD in the MPOA greatly reduced male sexual behavior without affecting aggressive behavior (Sano et al., 2013, 2016). These results clearly demonstrate differential roles of MPOA-ERβ from

MPOA-ERα in the regulation of male social behaviors.

Previous studies using βERKO mice suggested inhibitory role of ERβ in aggressive behavior (Ogawa et al., 1999; Nomura et al., 2002). However, the result of this experiment suggested that activation of ERβ may facilitate aggressive behavior. Direction of ERβ action suggested from this experiment is consistent with the previous study in which neonatal treatment of male rat with selective ERβ agonist Diarylpropionitrile increased aggressive behavior in adulthood (Patisaul and Bateman, 2008). Results in the present study demonstrated for the first time that expression of ERβ in the MPOA during and/or after pubertal period is necessary for full expression of aggressive behavior. It is possible that ERβ plays an inhibitory role in aggressive behavior in other brain site. Determination of brain site(s) in which ERβ inhibits male aggressive behavior is emerging question for future study.

In this experiment, expression of ERβ gene was suppressed starting from the pre-pubertal period. Thus, PP-MPOA-βERKD mice did not express ERβ in the MPOA throughout pubertal period and adulthood. It remains still unknown which of pubertal organizational action and adult activational action of testosterone via ERβ plays a critical role in facilitation of aggressive behavior. To answer this question, it is necessary to test the influence of MPOA-βERKD only in adulthood on aggressive behavior. In Chapter 4, effects of adult knockdown of ERβ in the MPOA on male social behaviors were further examined.

Pre-pubertal βERKD in the MeA affected neither sexual nor aggressive behaviors in

adulthood. These results suggest that ERβ in the MeA during the pubertal period and in adulthood may play a minor role in the performance of sexual and aggressive behavior. However, it is possible that ERβ in the MeA might have other role than the regulation of sexual and aggressive behaviors, e.g. social information processing. As described in the General Introduction, the MeA is known to play a pivotal role in social information processing necessary for the performance of male social behaviors (Ferguson et al., 2002; Baum, 2009; Dhungel et al., 2011). Moreover, it is likely that ERβ may have a role in social information processing related to opposite-sex individual (Kavaliers et al., 2008). Thus, it is necessary to investigate the role of ERβ in the MeA in social information processing. In Chapter 5, I examined effects of adult knockdown of ERβ in the MeA on social information processing assessed by sexual preference tests and social recognition tests.

-Chapter 4-

Experiment 2:

Effects of Adult ERβ Knockdown in the MPOA

4. Experiment 2: Effects of Adult ERβ Knockdown in the MPOA

4.1. Introduction

In Experiment 1, pre-pubertal knockdown of ERβ in the MPOA significantly reduced aggressive behavior without affecting sexual behavior. These results indicated that ERβ expression in the MPOA during pubertal period and/or adulthood is necessary for facilitation of aggressive behavior. However, it remains still unknown whether pubertal organizational action of ERβ plays a critical role or activational action of ERβ is sufficient for full expression of aggressive behavior in adulthood. To answer this question, it is necessary to test the influence of MPOA-βERKD only in adulthood on aggressive behavior. In Chapter 4, the effects of adult knockdown of ERβ in the MPOA on male social behaviors were further examined. Particularly, I aimed to investigate the effects of MPOA-βERKD only in adulthood on aggressive behavior in this experiment. By comparing the effects of pre-pubertal and adult βERKD, it is possible to determine the roles of pubertal and adult ERβ in the MPOA.

Unaltered sexual behavior by pre-pubertal βERKD in Experiment 1 suggested a minor role of pubertal and adult ERβ in the MPOA in the performance of male sexual behavior. However, previous studies have indicated that the MPOA may play an essential role in sexual behavior (Paredes, 2003; Veening et al., 2005; Hull and Rodoriguez-Manzo, 2009). The MPOA receives dopaminergic innervation and implicated in sexual motivation and performance (Hull et al., 1995, 1997). Lesions of the MPOA disrupt not only the performance of sexual behavior (Paredes, 2003; Hull and Rodoriguez-Manzo, 2009), but also male-type sexual preference toward a receptive female over a non-receptive female or a male (Dhungel et al., 2011). Although ERβ in the MPOA is not essential for the performance of sexual behavior, it is possible that ERβ may have a role in the regulation

of sexual preference in the MPOA. Therefore, in this experiment, sexual preference tests were conducted in addition to sexual and aggressive behavior tests.

4.2. Methods

Gonadally intact adult male mice (12.2±1.00 wks at the time of injection) were stereotaxically injected with either AAV-shERβ (MPOA-βERKD, n=11) or AAV-shLUC (MPOA-Cont, n=14). Coordinate was AP +0.02, ML ±0.5, DV -5.65. Experimental procedure is illustrated in Figure 15. One week after surgery, all mice were individually housed and a series of biweekly sexual (SEX) and aggressive (AGG) behavior tests described in Experiment 1 was started on the following week. After the last aggressive behavior test, all mice were tested for olfactory sexual preference tests twice, one with the PTFF and the other with the PTFM paradigm in this order. Minimum of five days was elapsed between the last aggression test and PTFF and between two preference tests. After the completion of behavioral tests, brain tissues were collected and processed for immunohistochemistry for GFP.

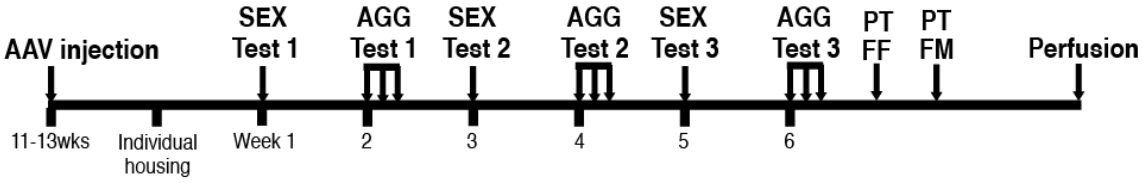

Figure 15. Schema of experimental procedures. Tick marks under the horizontal bar indicate one week. SEX, sexual behavior; AGG, aggressive behavior.

4.3 Results

Similar to the results of pre-pubertal knockdown, male sexual behavior was not altered in the MPOA-βERKD compared to MPOA-Cont groups (Figure 16). Statistical analyses revealed a significant increase of the number of mount ($F_{1.712,39.373} = 5.078$, $p <$

0.05; adjusted by Greenhouse-Geisser) and intromission ($F_{1.448,33.296} = 4.185$, $p < 0.05$; adjusted by Greenhouse-Geisser), and a decrease of latency to first mount ($F_{2,46} = 9.470$, $p < 0.01$) along the repeated sexual behavioral tests. However, there was no significant main effect of treatment and interaction of treatment and test in any of number of mounts (treatment: $F_{1,23} = 3.627$, $p = 0.069$; treatment x test: $F_{1.712,39.373} = 1.682$, *n.s.*; adjusted by Greenhouse-Geisser) and intromissions (treatment: $F_{1,23} = 2.562$, *n.s.*; treatment x test: $F_{1.448,33.296} = 1.547$, *n.s.*; adjusted by Greenhouse-Geisser), and latency to the first mount (treatment: $F_{1,23} = 3.434$, $p = 0.077$; treatment x test: $F_{2,46} = 1.954$, *n*.s.). These results indicated that ERβ knockdown in adult MPOA has minimal effects on sexual behaviors.

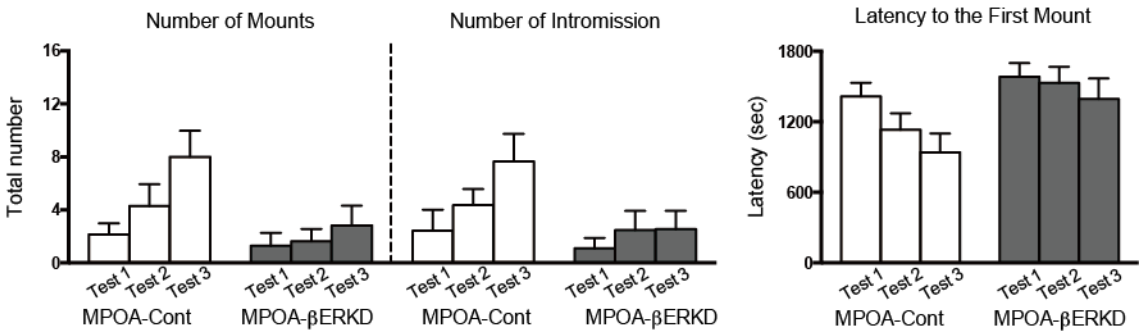

Figure 16. Effects of βERKD in adult MPOA on male sexual behavior. There were no difference between the MPOA-Cont and MPOA-βERKD groups in either number of mounts (left panel), intromissions (middle panel), or latency to the first mount (right panel). All data are presented as mean+SEM.

In aggressive behavior tests, unlike the observation in pre-pubertal MPOA groups in Experiment 1, MPOA-βERKD and MPOA-Cont groups showed equivalent levels of aggressive behaviors (Figure 17). Statistical analyses revealed a significant increase of the number ($F_{2,46} = 4.199$, $p < 0.05$) and duration ($F_{2,46} = 3.582$, $p < 0.05$) of aggressive bouts along the repeated tests. However, there was significant main effect of treatment and interaction of treatment and test in neither of number (treatment: $F_{1,23} = 0.033$, *n.s.*;

treatment x test: $F_{2,46} = 0.189$, *n.s.*) nor duration (treatment: $F_{1,23} = 0.009$, *n.s.*; treatment x test: $F_{2,46} = 0.229$, *n.s.*) of aggressive bouts. These results indicated that ERβ knockdown in adult MPOA did not affect male aggressive behavior.

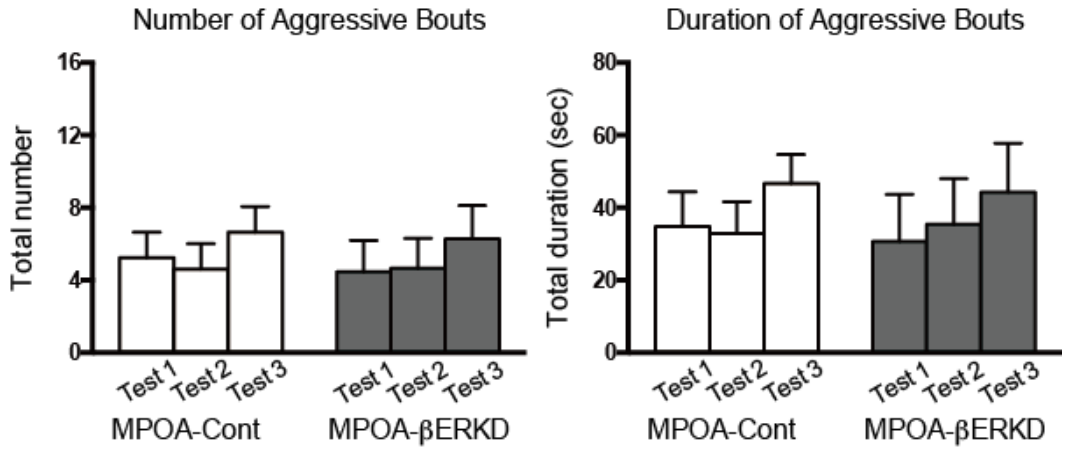

Figure 17. Effects of adult βERKD in the MPOA on male aggressive behavior in adulthood. There were no difference between the MPOA-Cont and MPOA-βERKD groups in either duration (left panel) or number (right panel) of aggressive bouts. All data are presented as mean+SEM.

In olfactory sexual preference tests, experimental animals were tested whether they preferred receptive females (RF) than non-receptive female (XF) in PTFF or intact male (IM) in PTFM (Figure 18). In both of PTFF and PTFM, MPOA-βERKD and MPOA-Cont groups showed significantly longer SI duration toward RF than toward XF in PTFF (βERKD: $t_{10} = 3.561$, $p < 0.01$; Cont: $t_{13} = 3.492$, $p < 0.01$) or toward IM in PTFM (βERKD: $t_{10} = 6.165$, $p < 0.01$; Cont: $t_{13} = 10.560$, $p < 0.01$). These results indicated that sexual preference toward RF was not disrupted in MPOA-βERKD males.

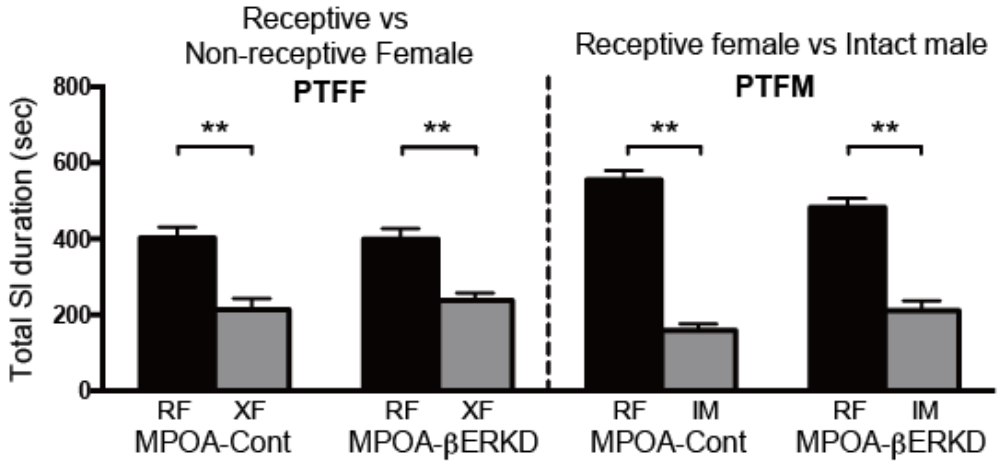

Figure 18. Effects of βERKD in adult MPOA on sexual preference. Both of the MPOA-Cont and MPOA-βERKD groups showed longer SI duration toward RF in PTFF (left panel) and PTFM (right panel) tests (**$p < 0.01$). All data are presented as mean+SEM.

Moreover, total durations of SI toward RF plus XF in PTFF, and toward RF plus IM in PTFM were not different between MPOA-βERKD and MPOA-Cont groups in both tests (Figure 19, PTFF: $t_{23} = 0.774$, *n.s.*; PTFM: $t_{23} = 0.688$, *n.s.*). These results indicated that the levels of social investigation toward two stimulus animals were not altered by adult βERKD in the MPOA.

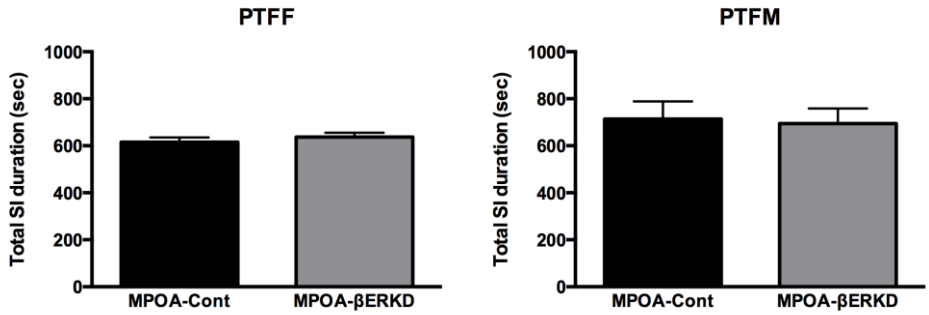

Figure 19. Effects of βERKD in adult MPOA on SI in olfactory sexual preference test. Total SI duration toward two stimulus animals did not differ between MPOA-Cont and MPOA-βERKD groups in PTFF (left panel) and PTFM (right panel) tests. All data are presented as mean+SEM.

The placement of the injection needle tip for each mouse was examined and depicted in Figure 20. All animals used in behavioral analysis were checked for distribution of GFP-immunopositive cells to confirm that AAV vector was successfully injected bilaterally within the MPOA.

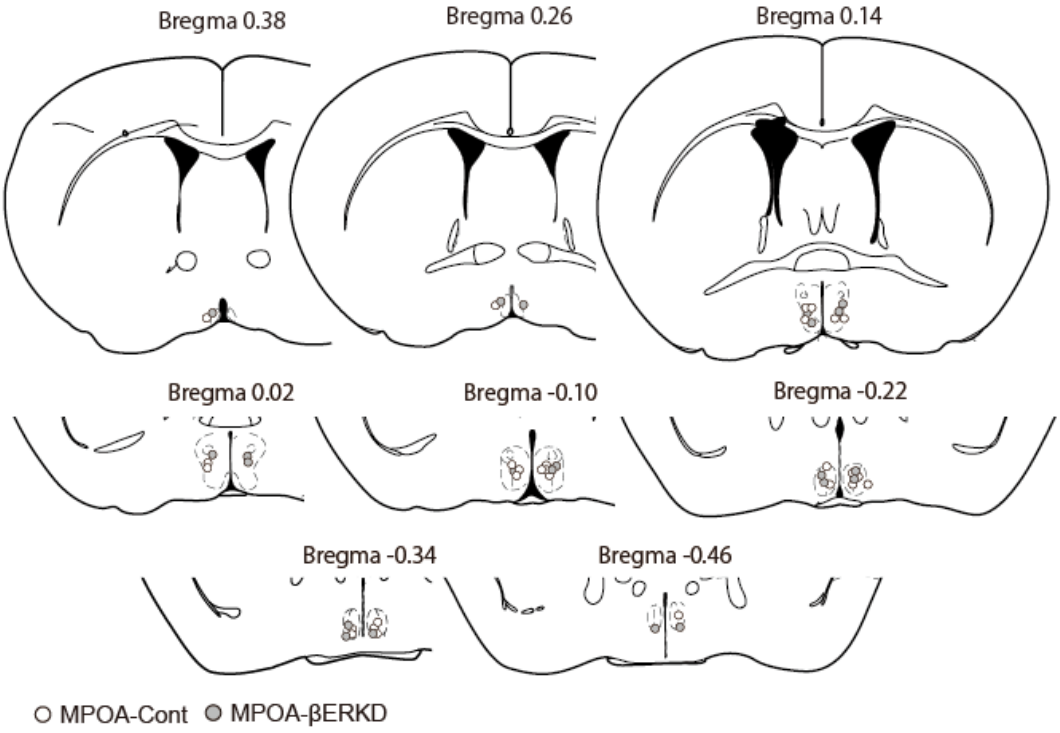

Figure 20. Histological diagrams depicting the placement of the injection needle tip for each mouse in the MPOA-Cont (open circles) and MPOA- βERKD (solid circles) groups.

4.4. Discussion

Suppression of ERβ gene expression in the MPOA only in adulthood did not affect any of sexual behavior, aggressive behavior, and male-type sexual preference. These results suggested that ERβ in adult MPOA plays a relatively minor role in male social behavior. Thus, activational action of testosterone through ERβ in the MPOA may not be necessary for full expression of male aggressive behavior.

Taken together with the results in Experiment 1, reduction of aggressive behavior in pre-pubertal, but not adult, MPOA-βERKD mice indicates that pubertal ERβ in the MPOA contributes to facilitation of male aggressive behavior. i.e. Pubertal ERβ in the MPOA may be involved in the formation and/or development of the neural network for aggressive behavior. A previous study has reported increased levels of aggressive behavior in pubertal βERKO mice indicating importance of ERβ during developmental period (Nomura et al., 2002). The results in the present study further demonstrated existence of ERβ-mediated pubertal organizational action of testosterone and identified the MPOA as one of critical brain sites involved. Importance of the MPOA in the neural network for male aggressive behavior has been implicated in previous studies (Veening et al., 2005; Wu et al., 2014). Possible roles of ERβ in the organization of social behavior neural networks will be further addressed in General Discussion (see 7.1.1.).

Unaltered sexual behavior and male-type sexual preference in MPOA-βERKD groups suggested that differential role of ERβ from that of ERα in the MPOA (Sano et al., 2013). ERα in adult MPOA is necessary for the performance of sexual behavior. On the other hand, ERβ in pubertal but not adult MPOA is necessary for facilitation of aggressive behavior. Underlying mechanism of these behavioral and temporal difference in the role of ERα and ERβ should be further investigated in future study.

-Chapter 5-

Effects of Adult ERβ Knockdown in the MeA

5. Effects of Adult ERβ Knockdown in the MeA

5.1. Introduction

Unaffected sexual and aggressive behavior in adulthood by pre-pubertal knockdown of ERβ in the MeA found in Experiment 1 suggest that expression of ERβ in the MeA during and after pubertal period may play a relatively minor role in these behaviors. Thus, it is predicted that βERKD only in adulthood may not affect sexual and aggressive behavior. However, the MeA has been implicated not only in the performance of sexual and aggressive behaviors but also in social information processing (Ferguson et al., 2002; Baum, 2009). As described in General Introduction (see 1.5.), the MeA receives innervation from olfactory systems and conveys social information to hypothalamic regions including the MPOA. Adequate social information processing is necessary for appropriate reaction to different types of opponents. For instance, gonadally intact male mice preferentially investigate a sexually receptive female when it is simultaneously presented with a non-receptive female and a male in sexual preference tests. It has been interpreted that preferential investigation reflects preference to a receptive female as a mating partner. Social odor information necessary for sexual preference is integrated and sorted out within the MeA (Ferguson et al., 2001, 2002) and sent to relevant brain sites for the performance of subsequent social behaviors (Choi et al., 2005; Swann et al., 2013). Disrupted male sexual preference by MeA lesions (Kondo and Sachs, 2002; Dhungel et al., 2011) also indicates the importance of the MeA in this process.

Not only sex and reproductive states, but also degree of familiarity of the opponent may alter social behavior of male rodents. Generally, repeated presentation of a same stimulus animal induces habituation to the stimulus in social recognition tests. Moreover, it is known that risk-taking behavior in male mice alters depending on familiarity of a

female exposed before the test. After exposure to a novel and receptive female mouse, WT male mice spent longer time in place with a predator odor compared to the test done after exposure to a familiar female (Kavaliers et al., 2008). It is well known that the MeA plays an essential role in the discrimination of opponents based on familiarity. However, ERβ in the MeA might not be necessary for the performance of social recognition test with habituation-dishabituation paradigm since it is reported that βERKO male mice do not show altered performance in social recognition test with a habituation-dishabituation paradigm using same-sex (male) stimulus mice (Sánchez-Andrade and Kendrick, 2011). However, Kavaliers et al. (2008) reported that unlike WT mice (see above), βERKO mice showed similar levels of risk-taking behavior after being exposed to novel and familiar receptive females. Thus, in male mice, ERβ might have a role in processing of social information relevant to receptive females. Considering importance of the MeA in the neural network for male social behaviors, it is possible that ERβ in the MeA is responsible for female-related information processing.

In Experiment 3, I intended to examine the influence of adult βERKD on social information processing using sexual preference test and social recognition test. Sexual preference tests can examine males' ability to discriminate two type of stimulus animals and their preference to attractive stimulus animal for normal gonadally intact males. In social recognition test, I aimed to test the effects of MeA-βERKD on the habituation and dishabituation to three types of stimulus animals; receptive female, non-receptive female, and gonadally intact male. It is possible that responses of MeA-βERKD mice to female stimuli might be altered. Moreover, it has been reported that mice showed longer investigation toward an opponent of different-sex than same-sex even when stimulus animal was presented separately (DiBenedictis et al., 2012). It is intriguing to investigate the effect of MeA-βERKD on social investigation toward different types of stimulus

animal. Social information processing, especially processing of information about female's reproductive states is necessary for male animals to choose an appropriate partner for subsequent sexual behavior. This is important for efficient reproduction since showing preference to a receptive female ensures choosing a female with high probability of pregnancy.

In Experiment 4, I aimed to additionally investigate the relationship of information processing of female odor (Experiment 3) and actual partner choice for the performance of sexual behavior. For this purpose, I performed 2F Sex test in which freely moving receptive and non-receptive females were simultaneously introduced to male's home?? cage. To examine the role of MeA-ERβ in social information processing and subsequent performance of sexual behavior provides evidence of relative importance of ERβ in the series of social behavior toward opposite-sex conspecifics.

5.2. Experiment 3: Effects of adult ERβ knockdown in the MeA on male-type sexual preference, sexual and aggressive behavior

5.2.1. Methods

Gonadally intact adult male mice were individually housed (9.7±0.49 wks). Experimental procedure is illustrated in Figure 21. Starting one week later, they were given an exposure session. Briefly, a hormonally primed receptive C57BL/6J female mouse was placed in a clear columnar Plexiglas cylinder (Mouse Cylinder SIOT1, see 2.3.4.) and presented in the center of the male's home cage for 30 minutes. Starting at least four days after the exposure session, experimental animals were transferred to white plastic testing cages and given two screening olfactory sexual preference tests, one with PTFF and the other with PTMF paradigms. Only the mice those showed longer SI toward a receptive female (RF) over non-receptive female (XF) (PTFF paradigm) and intact male

(IM) (PTMF paradigm) were selected. On 3-7 days after the completion of the screening tests, experimental mice were injected with either AAV-shERβ (MeA-βERKD, n=15) or AAV-shLUC (MeA-Cont, n=13). Coordinate was AP -1.7, ML ±2.4, DV -5.4. Three weeks after injections, all mice were given PTFF, PTFM, and PTMM (gonadectomized male: XM versus intact male: IM) olfactory sexual preference tests, social recognition tests (SR) with RF, XF, and IM with four days of intervals. Starting one week after the completion of social recognition tests, they were given three sexual behavior tests (SEX) and two sets of aggressive behavior tests (AGG) during the period of five weeks.

After the completion of behavioral tests described above, some of the experimental mice (MeA-βERKD, n=7, MeA-Cont, n=4) were tested additionally for sexual behavior toward a non-receptive female and preference between non-receptive female and intact male (Appendix). After the completion of the last behavioral test, brain tissues were collected and processed for immunohistochemistry for GFP.

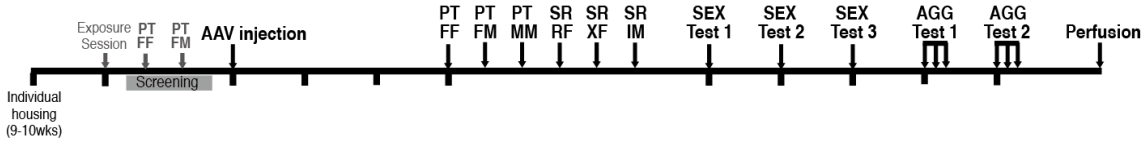

Figure 21. Schema of experimental procedures. Tick marks under the horizontal bar indicate one week. PT, olfactory sexual preference test; SR, social recognition test; SEX, sexual behavior; AGG, aggressive behavior.

5.2.2. Results

In olfactory sexual preference tests, MeA-βERKD mice showed disruption of male-type sexual preference. In the PTFF (Figure 22, right panel), MeA-Cont males investigated RF significantly longer than XF (t_{12} = 2.504; $p < 0.05$). However, MeA-βERKD males failed to show any preference in this test (t_{14}=0.199; p = 0.854, *n.s.*). On the other hand, in the PTFM (Figure 22, center panel), both of MeA-βERKD and MeA-

Cont groups showed significantly longer SI duration toward RF than toward IM (βERKD: $t_{14} = 7.446$; $p < 0.001$; Cont: $t_{12} = 4.534$; $p = 0.001$). These results indicate that βERKD in adult MeA disrupts male's sexual preference of receptive over non-receptive females without affecting sexual preference of receptive females, over intact males.

Moreover, both of MeA-βERKD and MeA-Cont groups showed significantly longer SI duration toward XM than toward IM in the PTMM (Figure 22, left panel, βERKD: $t_{14} = 4.009$; $p < 0.01$; Cont: $t_{12} = 2.465$; $p < 0.05$). These results indicate that MeA-βERKD in adult does not affect male's preference of gonadectomized over intact males.

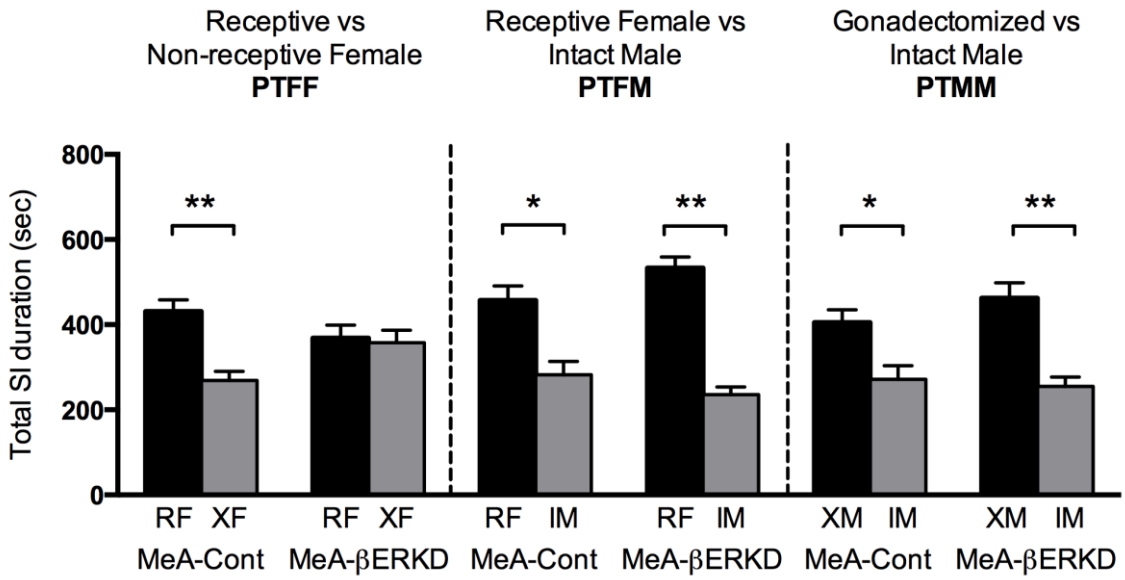

Figure 22. Effects of ERβ knockdown in adult MeA on male sexual preference. In the PTFF, unlike the MeA-Cont group, the MeA-βERKD group failed to show longer SI duration toward RF (left panel). Both of the MeA-Cont and MeA-βERKD groups showed longer SI duration toward RF in the PTFM (middle panel) and toward gonadectomized males in the PTMM (right panel) (*$p < 0.05$, **$p < 0.01$). All data are presented as mean+SEM.

Total durations of SI toward RF and XF in PTFF, toward RF and IM in PTFM, and toward XM and IM in PTMM were not different between MeA-βERKD and MeA-Cont groups in all tests (Figure 23, PTFF: t_{26}=0.743, *n.s.*; PTFM: t_{26}=0.987, *n.s.*; PTMM: t_{26}=0.960, *n.s.*). These results indicate that the level of total social investigation toward two stimulus animals is not affected by βERKD in the MeA.

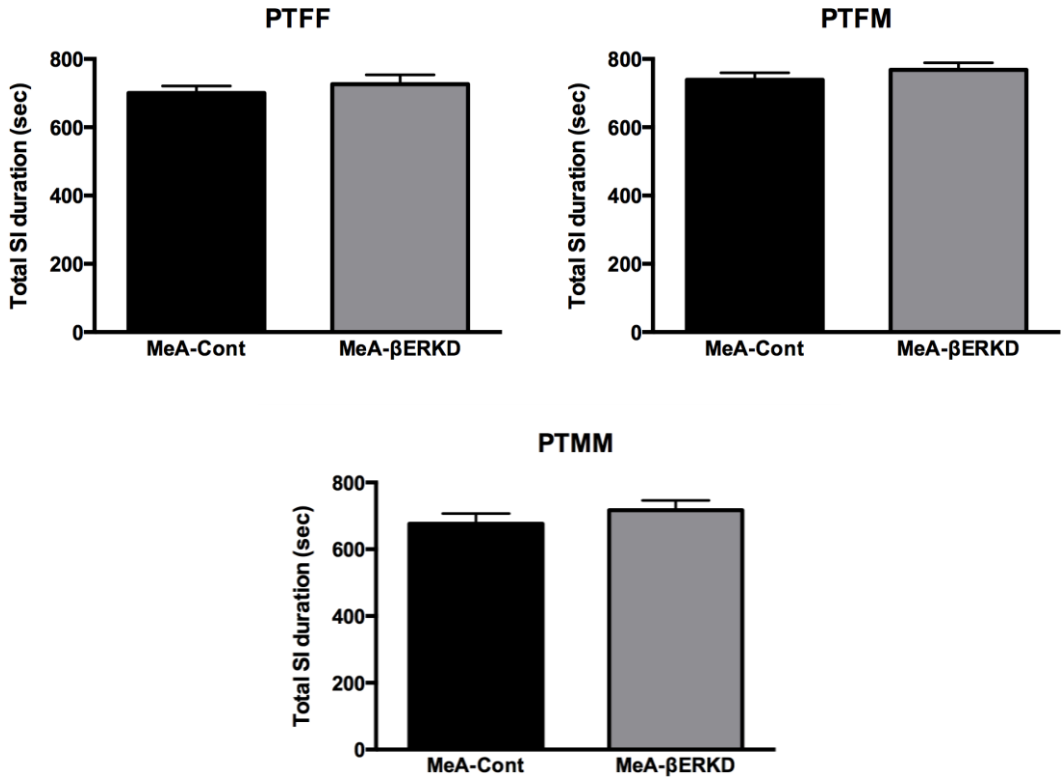

Figure 23. Effects of βERKD in adult MeA on SI in olfactory sexual preference test. Total SI duration of two stimulus animals did not differ between MeA-Cont and MeA-βERKD groups in PTFF (top left panel), in PTFM (top right panel) and in PTMM (bottom panel). All data presented as mean+SEM.

In social recognition tests with RF and with XF, both MeA-Cont and MeA-βERKD mice failed to show a significant change of SI duration along the repeated trials (Figure 24, top panels). Statistical analysis revealed that there were no significant main effects of

treatment and trial, and interaction of treatment and trial in the SI duration both RF test (treatment: $F_{1,26} = 0.016$, *n.s.*; trial: $F_{2.457,63.893} = 1.811$, *n.s.*; treatment x trial: $F_{2.457,63.893} = 0.489$, *n.s.*; adjusted by Greenhouse-Geisser) and XF test (treatment: $F_{1,26} = 0.002$, *n.s.*; trial: $F_{1.884,48.980} = 0.363$, *n.s.*; treatment x trial: $F_{1.884,48.980} = 0.253$, *n.s.*; adjusted by Greenhouse-Geisser). In these tests, expected habituation and dishabituation were not observed even in the MeA-Cont group since experimental animals, which were ICR/Jcl strain, showed long SI duration throughout four trials. These experimental mice also showed long SI duration in the preference test (about 700 sec in 900 sec of testing duration). This long SI duration might be characteristics of ICR/Jcl strain since C57B/6J strain in previous study (Tsuda et al., 2012), in which the same testing apparatus were used, SI duration was about 170 sec in the testing duration of 600 sec. Thus, it is hypothesized that ICR/Jcl male mice did not show a decline of SI duration to a familiar individual in such short trial duration as 240 sec because of their propensity of intensive social investigation.

On the other hand, in the social recognition tests with IM, SI duration changed along the repeated trials in both of MeA-Cont and MeA-βERKD groups (Figure 24, bottom panel). Statistical analysis revealed significant main effect of trial and interaction of treatment and trial (trial: $F_{3,75} = 13.179$, $p < 0.001$; treatment x trial: $F_{3,75} = 2.921$, $p < 0.05$). However, main effect of treatment was not significant ($F_{1,25} = 0.166$, *n.s.*). Both groups showed an increased SI to a novel stimulus mouse introduced in the trial 4 compared to other trial(s). Post hoc analysis revealed that, in MeA-Cont group, SI duration in the trial 4 was significantly longer than that in all the other trials ($p < 0.05$) and that, in MeA-βERKD group, SI duration in the trial 4 was significantly longer than that in the trial 3 only ($p < 0.05$). Both MeA-βERKD and MeA-Cont groups responded to a novel stimulus IM mouse with longer SI than to a familiar IM mouse. However,

difference in SI duration between the trial 1-3 (familiar stimulus) and the trial 4 (novel stimulus) was smaller in MeA-βERKD mice than that in MeA-Cont mice. These results collectively suggest that altered social investigation in MeA-βERKD mice may reflect their tendency of augmented reaction toward stimulus intact male mice.

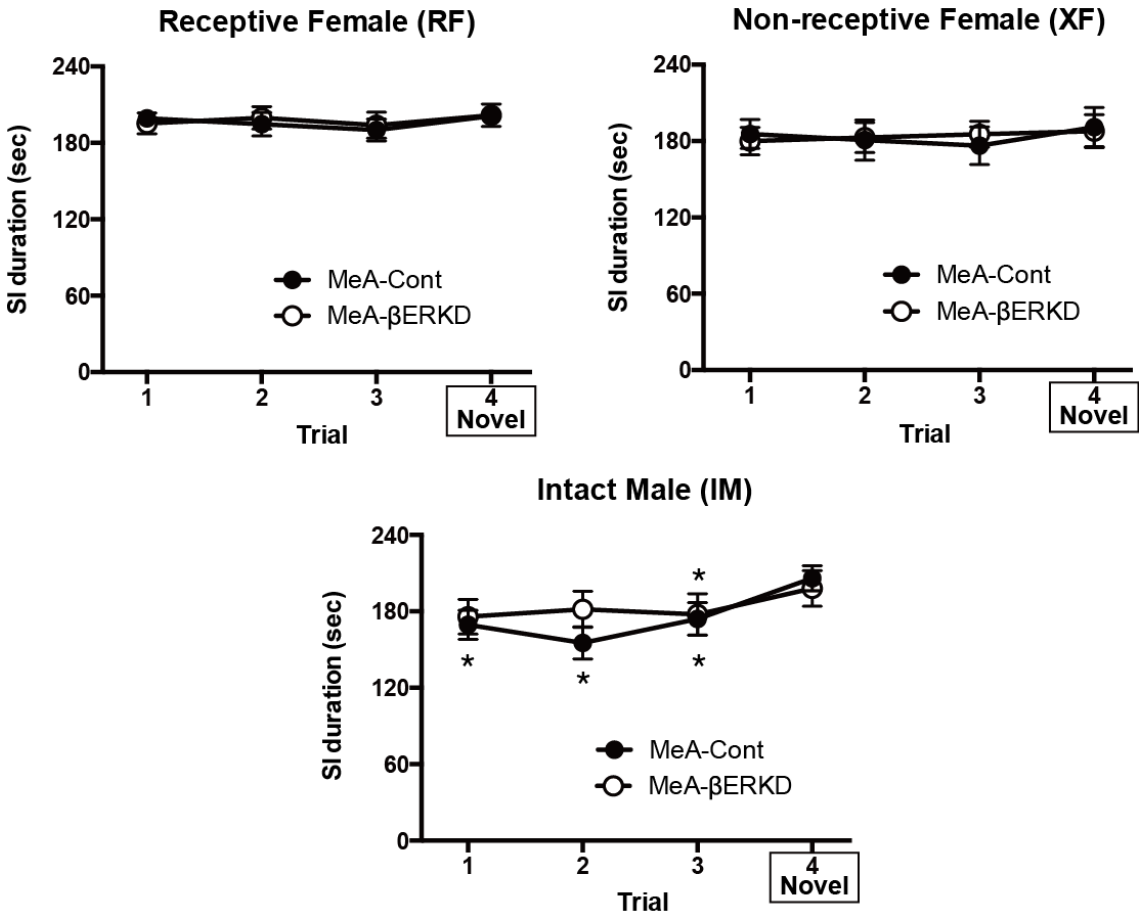

Figure 24. Effects of βERKD in adult MeA on SI in social recognition tests. SI duration of two stimulus animals did not differ between MPOA-Cont and MPOA-βERKD groups in RF test (top left panel), and in XF test (top right panel). In IM test (bottom panel), MPOA-βERKD group showed different SI changes along the repeated trials compared to MPOA-Cont group. *$p < 0.05$ vs trial 4 of same treatment group. All data are presented as mean±SEM.

As observed in pre-pubertal MeA groups in Experiment 1, MeA-βERKD in adulthood affected neither sexual nor aggressive behaviors. In sexual behavior tests (Figure 25), statistical analyses revealed a significant increase of the number of mount ($F_{2,48} = 7.780$, $p < 0.01$) and intromission ($F_{2,48} = 9.112$, $p < 0.01$), and a decrease of latency to the first mount ($F_{2,48} = 7.993$, $p < 0.01$) along repeated tests. However, there was no significant main effect of treatment and interaction of treatment and test in any of number of mounts (treatment: $F_{1,24} = 0.801$, *n.s.*; treatment x test: $F_{2,48} = 1.045$, *n.s.*) and intromissions (treatment: $F_{1,24} = 0.269$, *n.s.*; treatment x test: $F_{2,48} = 0.369$, *n.s.*), and latency to the first mount (treatment: $F_{1,24} = 0.057$, *n.s.*; treatment x test: $F_{2,48} = 0.842$, *n.s.*).

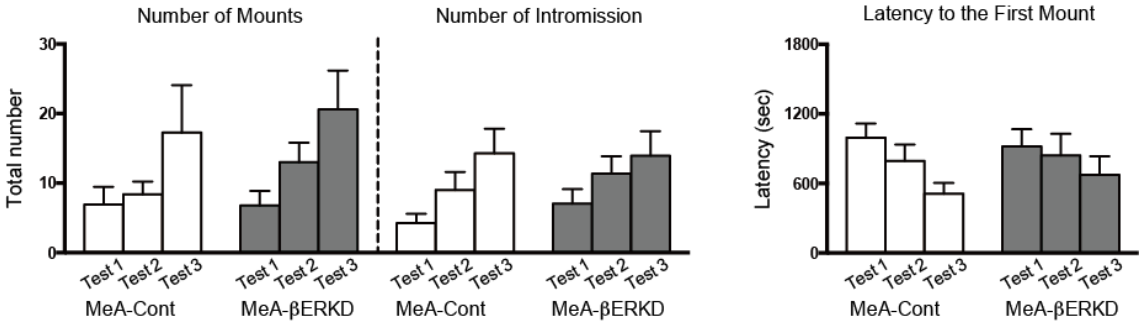

Figure 25. Effects of ERβ knockdown in adult MeA on sexual behavior. There was no difference between the MeA-Cont and MeA-βERKD groups in either number of mounts (left panel), intromissions (middle panel), or latency to the first mount (right panel). All data are presented as mean+SEM.

In aggressive behavior tests (Figure 26), there was no statistically significant main effects of treatment and test, and interaction of treatment and test in the number of aggressive bouts (treatment: $F_{1,25} = 1.316$, *n.s.*; test: $F_{1,25} < 0.001$, *n.s.*; treatment x test: $F_{1,25} = 0.022$, *n.s.*). In the duration of aggressive bouts, main effect of treatment ($F_{1,25} = 1.163$, *n.s.*) and interaction of treatment and test ($F_{1,25} = 0.679$, *n.s.*) were not significant

although significant main effect of test ($F_{1,25}$ = 4.678, p < 0.05) indicated a weekly decrease of the duration of aggressive bouts in both groups.

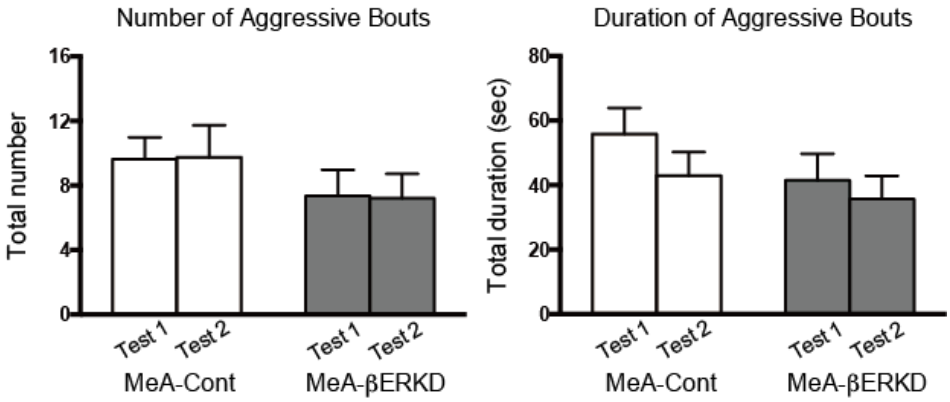

Figure 26. Effects of ERβ knockdown in adult MeA on aggressive behaviors. There was no difference between the MeA-Cont and MeA-βERKD groups in either duration (left panel) or number (right panel) of aggressive bouts. All data are presented as mean+SEM.

The placement of the injection needle tip for each mouse was examined and depicted in Figure 27. All animals used in the behavioral analysis were checked for distribution of GFP-immunopositive cells to confirm that AAV vector was successfully injected bilaterally within the MeA.

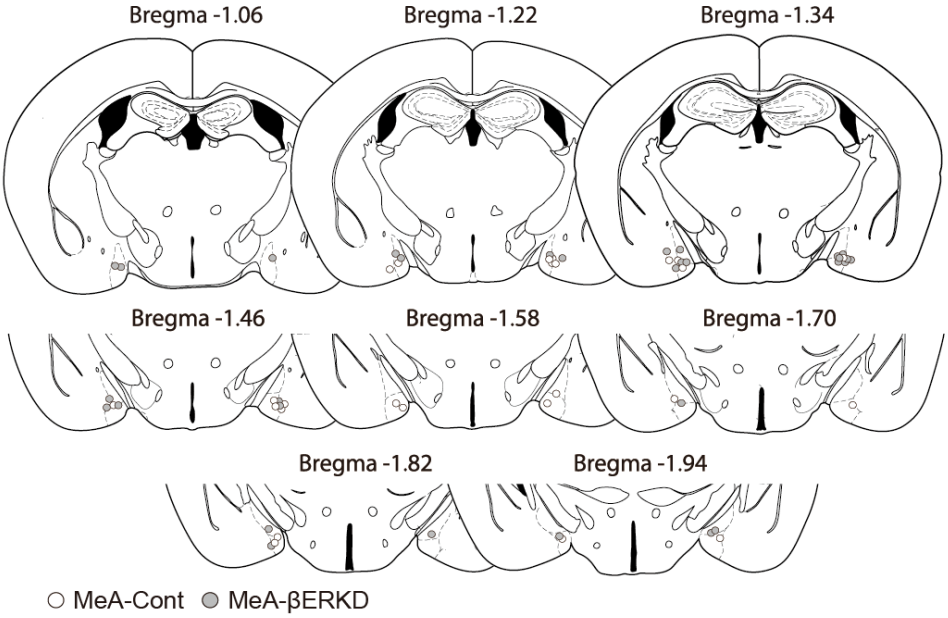

Figure 27. Histological diagrams depicting the placement of the injection needle tip for each mouse in the MeA-Cont (open circles) and MeA- βERKD (solid circles) groups.

5.2.3. Conclusions

Site-specific knockdown of ERβ in the MeA disrupted male's preference to RF over XF without affecting preference to RF over IM and preference to XM over IM. These results suggeste that ERβ in the MeA may be involved in the information processing for discrimination of female's receptivity and/or preferential investigation toward RF but not discrimination between males and females or discrimination of gonadal states of same-sex conspecifics. It is suggested that ERβ may play an essential role in social information processing and approaching to an appropriate female for efficient reproduction. To examine whether deficits of social information processing in MeA-βERKD mice actually affect subsequent performance of sexual behavior, I conducted the 2F Sex test in which males were allowed direct physical contact with freely moving RF and XF in Experiment 4.

In social recognition tests, βERKD in the MeA altered responses toward repeatedly introduced intact male stimulus animals although the effect of βERKD in the MeA on ability of social recognition and social memory could not be elucidated in this experiment. It is suggested that alteration of SI toward IM in MeA-βERKD mice may be consistent with phenotype reported in βERKO mice which show hyper-reactivity to same-sex stimulus animals (Handa et al., 2012; Tsuda et al., 2014).

As expected from the results of Experiment 1, the performance of sexual behavior toward a receptive female and aggressive behavior tested using resident-intruder paradigm was not affected by βERKD in adult MeA.

5.3. Experiment 4: Effects of adult ERβ knockdown in the MeA on sexual preference test with freely moving two females (2F Sex test)

5.3.1. Methods

Gonadally intact adult male mice (14.8±2.60 wks at the time of injection) were stereotaxically injected with either AAV-shERβ (MeA-βERKD, n=9) or AAV-shLUC (MeA-Cont, n=6). Coordinate was same as Experiment 3. Experimental procedure is illustrated in Figure 28. One week after the surgery, all mice were individually housed and sexual behavior test (SEX) was started on the following week. After two trials of sexual behavior tests, all mice were tested for the 2F sex test. After the completion of behavioral tests, brain tissues were collected and processed for immunohistochemistry for GFP. Animals used in behavioral analyses were mice that showed sexual behavior at least one mount or intromission in either trial of sexual behavior test and at least one mount or intromission toward either of RF or XF in the 2F Sex test.

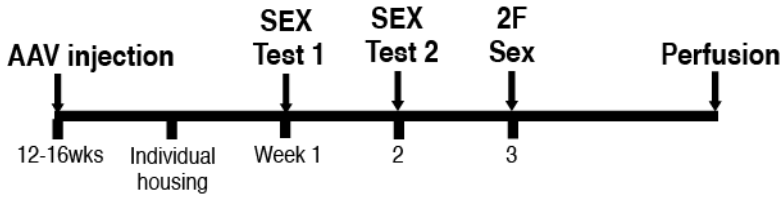

Figure 28. Schema of experimental procedures. Tick marks under the horizontal bar indicate weeks. SEX, sexual behavior; 2F Sex, 2F Sex test.

5.3.2. Results

As expected from the results in Experiments 1 and 3, sexual behavior was not altered by knockdown of MeA-ERβ in adulthood. In sexual behavior tests (Figure 29), statistical analysis revealed a significant increase of the number of intromission ($F_{1,13} = 14.680$, $p < 0.01$) along repeated tests. However, there was no significant main effect of test in number of mounts ($F_{1,13} = 1.877$, *n.s.*) and latency to the first mount ($F_{1,13} = 4.168$, $p = 0.062$).

Furthermore, there was no significant main effects of treatment and interaction of treatment and test in neither of number of mounts (treatment: $F_{1,13} = 2.026$, *n.s.*; treatment x test: $F_{1,13} = 3.067$, *n.s.*) and intromissions (treatment: $F_{1,13} = 0.002$, *n.s.*; treatment x test: $F_{1,13} = 0.250$, *n.s.*), and latency to the first mount (treatment: $F_{1,13} = 0.053$, *n.s.*; treatment x test: $F_{1,13} = 1.288$, *n.s.*).

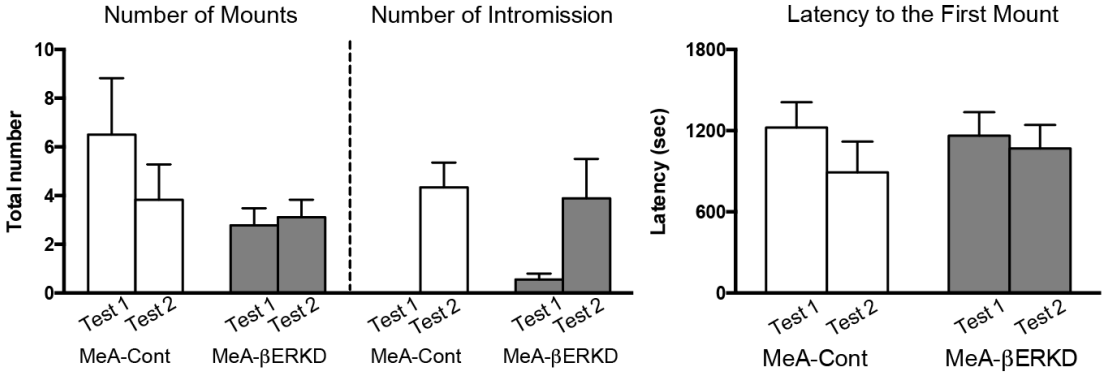

Figure 29. Effects of βERKD in adult MeA on sexual behavior. There were no differences between the MeA-Cont and MeA-βERKD groups in either number of mounts (left panel), intromissions (middle panel), or latency to the first mount (right panel). All data are presented as mean+SEM.

In the 2F Sex test, all experimental animals used in the behavioral analysis mounted to both RF and XF at least once. Moreover, all mice except one animal in the MeA-βERKD group showed intromission toward RF. Numbers of animals showed intromission toward XF were 3 out of 6 mice in the MeA-Cont group and 5 out of 9 mice in the MeA-Cont group (*n.s.* in Fisher's Exact Test). In this test, latency to the first mount and intromission toward each stimulus female, RF or XF, were used as index of partner choice for actual performance of sexual behavior (Figure 30). Although both of the MeA-βERKD and MeA-Cont group mounted with similar latency toward RF and XF (βERKD: $t_8 = 0.562$; *n.s.*; Cont: $t_5 = 1.774$; *n.s.*), MeA-Cont mice showed intromission toward RF

with shorter latency toward RF than toward XF (t_5 = 3.211; p < 0.05). These results indicate that MeA-Cont males choose RF as a partner of their sexual behavior. However, intromission latency toward RF and XF did not differ in MeA-βERKD mice (t_8 = 0.673; *n.s.*). These results indicate that βERKD in adult MeA disrupts partner choice in the situation of actual sexual behavior.

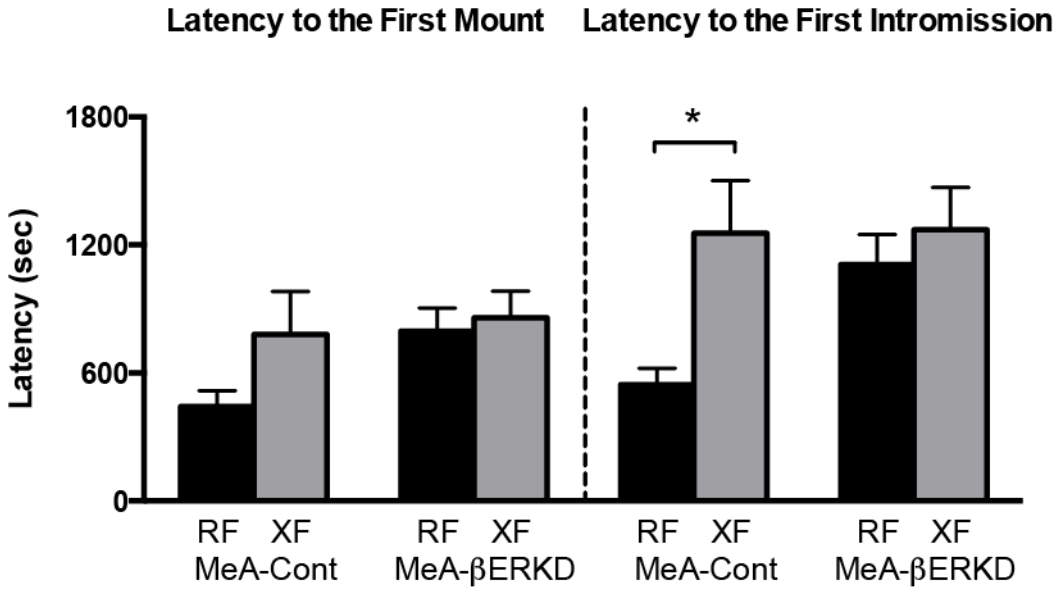

Figure 30. Effects of βERKD in adult MeA on sexual behavior latency in the 2F Sex test. In both of the MeA-Cont and MeA-βERKD groups, latency to the first mount did not differ between stimulus females (left panel). Latency to the first intromission toward RF is significantly shorter compared with that toward XF in MeA-Cont, but not in MeA-βERKD group (*p < 0.05). All data are presented as mean+SEM.

The placement of the injection needle tip for each mouse was examined and depicted in Figure 31. All animals used in the behavioral analysis were checked for distribution of GFP-immunopositive cells to confirm that AAV vector was successfully injected bilaterally within the MeA.

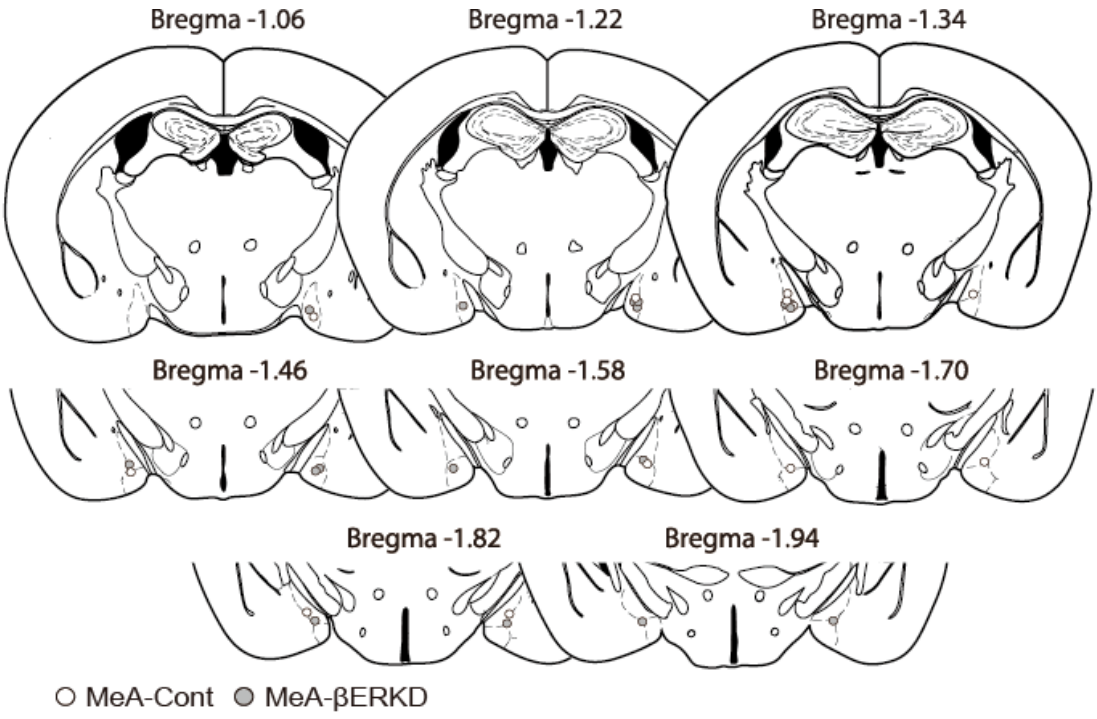

Figure 31. Histological diagrams depicting the placement of the injection needle tip for each mouse in the MeA-Cont (open circles) and MeA- βERKD (solid circles) groups.

5.3.3. Conclusions

In the 2F Sex test, MeA-βERKD mice failed to choose a receptive female as a partner of sexual behavior although their performance of sexual behavior toward a receptive female mouse in sexual behavior tests was unaffected. These results were consistent with the disrupted male-type sexual preference found in the PTFF in Experiment 3. It was suggested that βERKD in the MeA disrupted partner choice for actual sexual behavior in the situation of simultaneous introduction of receptive and non-receptive females. Thus, it can be concluded that disruption of olfactory sexual preference of receptive female over non-receptive female caused by MeA-βERKD actually affected partner choice for subsequent sexual behavior.

5.4. Discussion

In this chapter, I intended to examine the role of ERβ in adult MeA in social information processing. Firstly, I tested effects of βERKD in adult MeA on information processing about female's receptivity, sex difference, and male's gonadal states using PTFF, PTFM, and PTMM tests. As a result, adult βERKD in the MeA disrupted male-type sexual preference in olfactory sexual preference test between receptive and non-receptive females. Disrupted preferential SI without a drastic increase or decrease of total SI duration suggested disturbance of discrimination between receptive and non-receptive females without affecting sexual motivation and social interest. This hypothesis is consistent with unaffected preference of a receptive female over an intact male and performance of sexual behavior in MeA-βERKD mice. Minor role of ERβ in sexual motivation is also suggested by unaffected sexual preference of soiled bedding from receptive female over from intact male in βERKO male mice (Kudwa et al., 2005). To further confirm the ability of MeA-βERKD mice to discriminate female from male mice, preference test with XF and IM (PTXFIM) was additionally conducted after Experiment 3 (Figure A1) with limited number of experimental animals. In the PTXFIM test, MeA-βERKD group showed a significant longer SI duration toward XF than toward IM. This result further supported the notion that ERβ in the MeA may play a relatively minor role in discrimination between females and males. Collectively, ERβ in adult male MeA may be necessary for discrimination of female's receptivity but not of sex, and for preferential investigation of anesthetized female with hormonal priming.

As described in Introduction of this chapter (see 5.1.), previous studies indicated a pivotal role of the MeA in processing of social odor information. Disrupted sexual preference of receptive over non-receptive females was reported in male rats with a small lesion in the MeA (Kondo and Sachs, 2002). Recently, it is revealed that MeA lesions

disrupt preference of receptive over non-receptive females without affecting preference of a receptive female over an intact male (Dhungel et al., 2011). Consistency of effects of MeA lesions with current results suggest that ERβ in the MeA may be responsible for information processing about female receptivity. Kavaliers et al. (2008) reported that, unlike WT littermates, βERKO male mice showed an equivalent level of risk-taking after exposure to a familiar receptive female compared to their responses to a novel receptive female. Taken together with the results in the present experiments, ERβ in male mice may be necessary for a choice of more profitable female for sexual behavior, that is, receptive rather than non-receptive and novel rather than familiar. Results of this experiment indicate that at least the former action is dependent on ERβ in the MeA.

To demonstrate influence of social information processing mediated by MeA-ERβ on partner choice of actual sexual behavior, we conducted the 2F Sex test in Experiment 4. Control animals showed shorter latency to the first intromission toward RF than that toward XF. However, latency to the first intromission of MeA-βERKD mice did not differ between RF and XF. Thus, βERKD in the MeA disrupted not only preferential investigation toward a receptive female presented in the cylinder, but also preferential copulation with a receptive female in freely moving setup.

In the 2F Sex test in Experiment 4, MeA-βERKD mice showed equivalent levels of total number of sexual behaviors toward two females as MeA-Cont mice. Moreover, in sexual behavior tests toward non-receptive (OVX) female, there was no difference in sexual behavior between MeA-βERKD and MeA-Cont group (Figure A2). These results also suggest unaltered motivation and performance of sexual behavior toward non-receptive female in MeA-βERKD males. Collectively, it is suggested that ERβ in the MeA may have an important role in the regulation of social information processing about females' receptivity for efficient reproduction rather than in the regulation of sexual

motivation.

In social recognition tests with intact male stimulus mice, MeA-βERKD mice showed altered SI duration which might reflect augmented reactivity to a stimulus IM mouse in MeA-βERKD mice. It is consistent with hyper-reactivity to a same-sex stimulus mouse in the situation of social investigation without direct physical contact in βERKO male (Handa et al., 2012) and female (Tsuda et al., 2014) mice. In the PTMM of Experiment 3, MeA-βERKD mice did not show alteration of total SI duration toward IM and XM. Significant preference toward XM in MeA-βERKD mice in the PTMM suggest that they are able to discriminate stimulus male's gonadal states. Collectively, in MeA-βERKD males, responsibility to sexually active but not sexually inactive (such as gonadectomized) male stimulus mice was affected. Thus, it is suggested that ERβ has a role in the regulation of social interaction with sexually active males and ERβ in the MeA may be involved in the regulation of non-aggressive aspect of inter-male social interaction such as social investigation. However, very slight alteration of SI duration in social recognition test in MeA-βERKD mice test suggest that ERβ in the MeA may take charge of not very large part of the regulation of social interaction between males.

Moreover, ability to respond to a novel male stimulus mouse in social recognition tests suggests that unaltered social recognition and social memory in MeA-βERKD mice. Corresponding to previous report of unaltered social recognition ability in βERKO males (Sánchez-Andrade and Kendrick, 2011), it is suggested that ERβ in the MeA may play only a minor role in social information processing related to same-sex conspecifics.

-Chapter 6-

Experiment 5

Influence of Systemic Deletion of ERβ Gene on Repeated Social Interaction

6. Experiment 5: Influence of Systemic Deletion of ERβ Gene on Repeated Social Interaction

6.1. Introduction

In Experiments 1-4, site- and age-specific roles of ERβ on male social behaviors have been investigated. These results suggest that ERβ in the MPOA and MeA regulates different aspects of male social behaviors and acts at different age. The roles of ERβ in these brain sites may not be to turn on and off of stereo typical social behaviors but to modulate expression levels of these behaviors depending on the situation and the opponent.

As described in Chapter 5, ERβ might exert different roles in the regulation of social behaviors toward females and toward males. That is, ERβ in the MeA is necessary for discrimination of female's sexual receptivity and subsequent partner choice. On the other hand, multiple role of ERβ in the regulation of behaviors toward male opponent has been indicated in this and previous studies. Partially elevated aggressive behavior in βERKO mice and pubertal organization of neural network for aggressive behavior through ERβ in the MPOA (see Chapters 3 and 4) suggest roles of ERβ in the regulation of aggressive behavior. Moreover, ERβ may be involved in the regulation of social reactivity to an intact male stimulus (Handa et al., 2012) in which ERβ in the MeA may be partially involved (see Chapter 5). Moreover, a previous study using a selective ERβ agonist revealed that activation of ERβ in gonadally intact male mice increases dominance/agonistic behaviors toward the intruder including aggressive grooming and pushing down without affect attack in resident-intruder test (Allen et al., 2010). Thus, to investigate the role of ERβ in inter-male social interaction, it may be necessary to analyze agonistic interaction including non-aggressive behaviors.

It is known that male rodents establish dominant hierarchy through social interaction. Establishment of hierarchical social relationship can be observed various species from fishes to mammals. Hierarchical social relationship serves to maintain order within a group and to avoid severe damage by a conflict (Kaufmann, 1983). Considering biological meaning of the role of ERβ in inter-male social behaviors, including regulation of social investigation and aggressive behavior, these behavioral components regulated by ERβ may contribute to establish long-term social relationship including dominance hierarchy.

In this chapter, I aimed to investigate the role of ERβ in the modulation of establishment of inter-male social relationship. Although the roles of ERβ have been investigated in the situation of short-term social interaction like single episode of aggressive encounter, the roles of ERβ in long-term social relationship is still unclear. Thus, it is necessary to examine how ERβ-regulated behavioral components affect establishment of social relationship with same-sex conspecifics. In Experiment 5, influence of systemic deletion of ERβ gene on males' behavior in repeated agonistic interaction with same individual and on their establishment of dominance hierarchy was examined to initially investigate a role of ERβ in long-term social relationship. Unaffected social recognition ability in male βERKO mice (Sánchez-Andrade and Kendrick, 2011) suggest that they are able to recognize and memorize their partner. The reason of the use of βERKO mice was that it was likely that multiple brain sites might cooperatively regulate inter-male social interaction considering the effect of MeA-βERKD was rather subtle.

As described in General Introduction, a series of testing paradigms to investigate social relationship has been developed. In this experiment, I used "tube test" to examine the effect of ERβ gene deletion on establishment of social relationship in a pair of male

mouse. In the tube test, two mice run through a tube face-to-face and one mouse (winner) can push the other (loser) out from the end of the tube like “Don-Janken game” well-known among Japanese children. It is confirmed that score of the tube test highly correlates with that of other behavioral tests for evaluation of dominance hierarchy (Wang et al., 2011).

6.2. Methods

Gonadally intact adult male βERKO (KO) mice and their wild-type (WT) littermates were used as experimental animals (KO: n= 24, WT: n=20. 16.9±4.53 wks at start). Figure 32 illustrates experimental procedures. At the beginning of the experiment, animals were individually housed in small transparent plastic experimental home-cages (12.5x20x11 cm, CLEA Japan, Inc., Japan). Each Animal was paired with an unfamiliar experimental mouse of same genotype and matched body weight (± 3.5g). In behavioral tests, animals were tested against the same partner throughout the experiment. Each animal was kept in individual housing and met its partner only during behavioral tests. After one week of individual housing, training trial for tube test was started. Day 1 of behavioral tests was the next day of second training day. On testing day 1, 3, 5, 7, each pair was observed for agonistic behavior in neutral cage for 15 min. Immediate after agonistic behavior test, they underwent tube test.

Data from 12 KO pairs and 10 WT pairs was used for behavioral analysis. In day 1, three pairs (2 of WT and 1 of KO pairs) failed to complete testing trial. These data were excluded from the analyses of trial number. Moreover, all data from these pairs was excluded from analysis of latency to loser ejection.

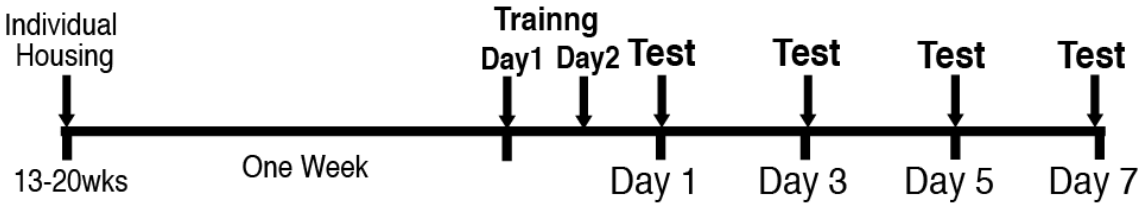

Figure 32. Schema of experimental procedures. Tick marks under the horizontal bar indicate 2 days.

6.3. Results

Firstly, the cumulative number and duration of agonistic and prosocial interaction in agonistic behavior tests were analyzed. Agonistic interaction includes aggressive behavior, fleeing, and tail rattling, and prosocial interaction includes sniffing, grooming, approaching, and huddling. In WT but not in KO mice, number of agonistic interaction increased with repeated trials (Figure 33, left panel). Statistical analysis revealed that there were significant main effects of genotype and day, and interaction of genotype and day in the number of agonistic interaction (genotype: $F_{1,42} = 13.203$, $p = 0.001$; day: $F_{2,307,96.877} = 7.852$, $p < 0.001$; genotype x day: $F_{2,307,96.877} = 4.683$, $p < 0.01$; adjusted by Greenhouse-Geisser). Post hoc analysis revealed that, on day 5 and 7, KO mice showed less agonistic interactions compared to WT mice ($p = 0.001$). Moreover, WT but not KO mice showed a significant increase of agonistic interactions on days 5 and 7 compared to day 1 ($p < 0.001$) and on days 5 compared to day 3 ($p < 0.05$). On the other hand, KO mice showed shorter overall duration of agonistic interaction compared to WT mice (Figure 33, right panel). Statistical analysis revealed that there was a significant main effect of genotype in the duration of agonistic interaction ($F_{1,42} = 8.437$, $p < 0.01$) but main effect of day and interaction of genotype and day were not significant (day: $F_{1,792,75.256} = 0.660$, *n.s.*; genotype x day: $F_{1,792,75.256} = 1.326$, *n.s.*; adjusted by Greenhouse-Geisser).

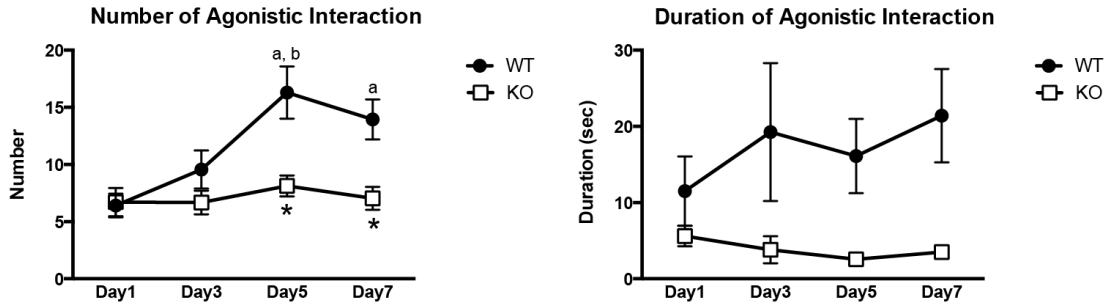

Figure 33. Genotype differences in number and duration of agonistic interaction. Change of agonistic interaction number (left panel) along repeated trial was different between βERKO (KO: empty square) and WT (WT: solid circle) mice. Moreover, βERKO mice showed shorter overall duration of agonistic behavior compared to WT mice (right panel). *: $p < 0.01$ vs WT, a: $p < 0.001$ vs Day 1 of the same genotype, and b: $p < 0.05$ vs Day 3 of same genotype. All data are presented as mean±SEM.

On the other hand, there was no genotype difference in the number and duration of prosocial interaction (Figure 34). Statistical analysis revealed that there were no significant main effects of genotype and day, and interaction of genotype and day in the number of prosocial interaction (genotype: $F_{1,42} = 0.082$, *n.s.*; day: $F_{2,434,102.214} = 1.078$, *n.s.*; genotype x day: $F_{2,434,102.214} = 0.704$, *n.s.*; adjusted by Greenhouse-Geisser). Similar to the duration of prosocial interaction, there were no significant main effects of genotype and day, and interaction of genotype and day (genotype: $F_{1,42} = 0.900$, *n.s.*; day: $F_{2,287,96.040} = 0.987$, *n.s.*; genotype x day: $F_{2,287,96.040} = 0.595$, *n.s.*; adjusted by Greenhouse-Geisser).

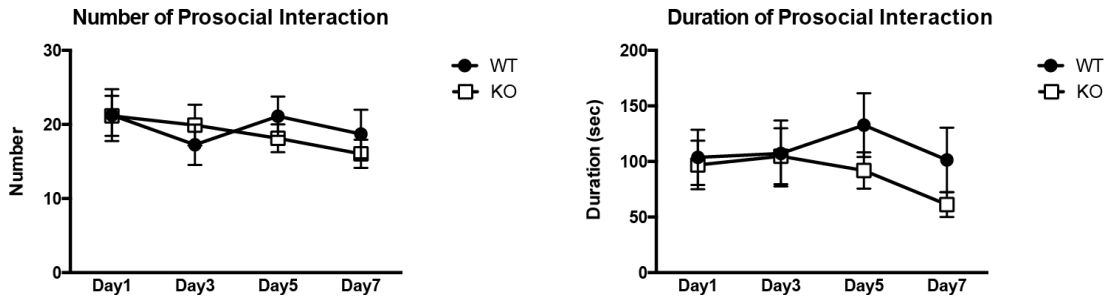

Figure 34. Genotype differences in number and duration of prosocial interaction.
Change of prosocial interaction number (left panel) and duration (right panel) were different in βERKO (KO: empty square) and WT (WT: solid circle) mice. All data are presented as mean±SEM.

In tube tests (Figure 35), the latency to loser ejection decreased with the repeated trials in WT mice. However, βERKO mice did not show a radical decrease of loser ejection latency along the repeated trials. Statistical analysis revealed that there were significant main effect of day, and interaction of genotype and day in the latency to loser ejection (day: $F_{2,325,88.334} = 18.677$, $p < 0.01$; genotype x day: $F_{2,325,88.334} = 8.551$, $p < 0.01$; adjusted by Greenhouse-Geisser). However, main effect of genotype was not significant ($F_{1,38} = 0.923$, *n.s.*). Post-hoc analysis revealed that βERKO mice showed significantly shorter latency to loser ejection in day 1, and longer latency in days 3, 5, and 7 than WT mice showed ($p < 0.05$). Moreover, only WT mice showed a significant decrease of latency on days 3, 5, and 7 compared to day 1 ($p < 0.01$).

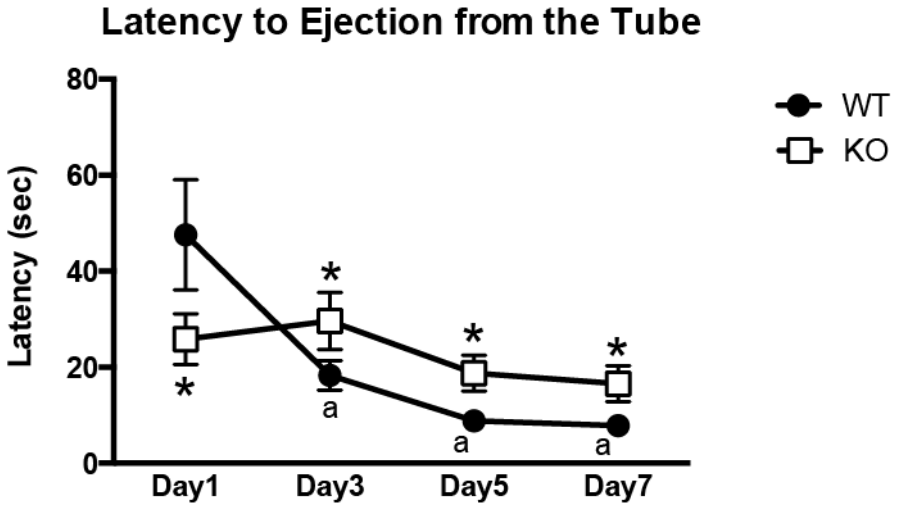

Figure 35. Genotype differences in number and duration of agonistic interaction.
Change of the latency to the loser ejection from the tube along the repeated trials was different between βERKO (KO: empty square) and WT (WT: solid circle) pairs. *: $p < 0.01$ vs WT, and a: $p < 0.01$ vs Day 1 of the same genotype. All data are presented as mean±SEM.

Figure 36 shows individual results of agonistic behavior tests and tube tests. Left columns in each day show aggression in the agonistic behavior test. “W” in a red box indicates that a winner but not a loser in tube test on the same day showed aggression in agonistic behavior test. “L” in a blue box similarly indicates aggression only by a loser. Moreover, “B” indicates aggression by both animals. In WT pairs, trials with “aggression by both mice (both)” mostly appeared on days 5 and 7. However, in KO pairs, the “both” trials were rare and not necessarily apparent during the latter half of experiment. Statistical analysis revealed that the proportion of “both” trials in all trials was larger in WT mice (12/40 trials) compared to that in βERKO mice (5/48 trials, $p < 0.05$ vs WT). Additionally, there was no genotype difference in the proportion of trials with aggression by winner only (winner trial) or trials with aggression by loser only (loser trial) out of all

trials (winner trial; WT: 5/40 trials, KO: 4/48 trials, *n.s.*; loser trial; WT: 9/40 trials, KO: 8/48 trials, *n.s.*).

Right columns in each day show events in the tube test. Black solid box indicates that test was not completed in that trial (missing data; tube test failure). A slash in the box indicates that the winner was different from that in the previous tube test (i.e., "winner change"). Statistical analysis revealed that there was no genotype difference in the proportion of trials "with winner" change out of all trials (WT: 10/38 trials, KO: 10/47 trials, *n.s.*). In WT pairs, these trials with "winner change" often occurred following to aggression in agonistic behavior test on the same day (7/10 trials). In KO pairs, on the other hand, "winner change" rarely occurred following to aggression in agonistic behavior test on the same day (2/10 trials, $p = 0.0698$ vs WT). Gray box indicates invasion by a winner. There was no difference in frequency of invasion between KO and WT pairs (WT: 18/38 trials, KO: 13/47 trials, $p = 0.0728$). Figure 36 demonstrates that frequency of invasion in WT pairs did not change throughout experiment whereas that in KO pairs was different from day to day.

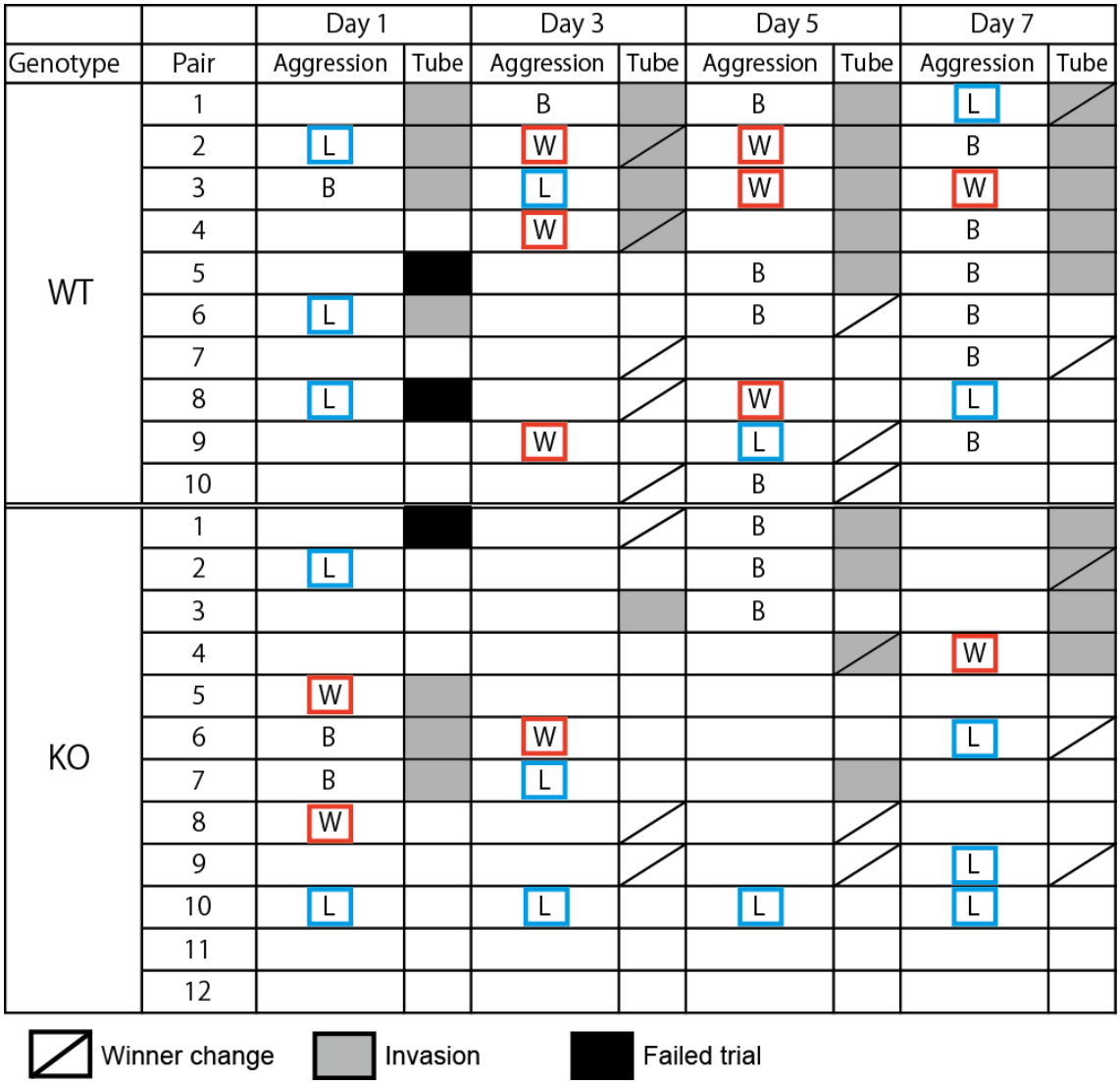

Figure 36. Individual results of agonistic behavior tests and tube tests.

Furthermore, relationship between the occurrence of invasion and the latency to loser ejection was analyzed (Figure 37). In both genotypes, loser ejection latency was longer in the trials with invasion than that in the trials without invasion. Statistical analysis revealed that main effect of invasion was significant ($F_{1,81} = 4.875$, $p < 0.05$) although main effect of genotype and interaction of genotype and invasion were not significant (genotype: $F_{1,81} = 1.060$, *n.s.*; genotype x invasion: $F_{1,81} = 0.263$, *n.s.*).

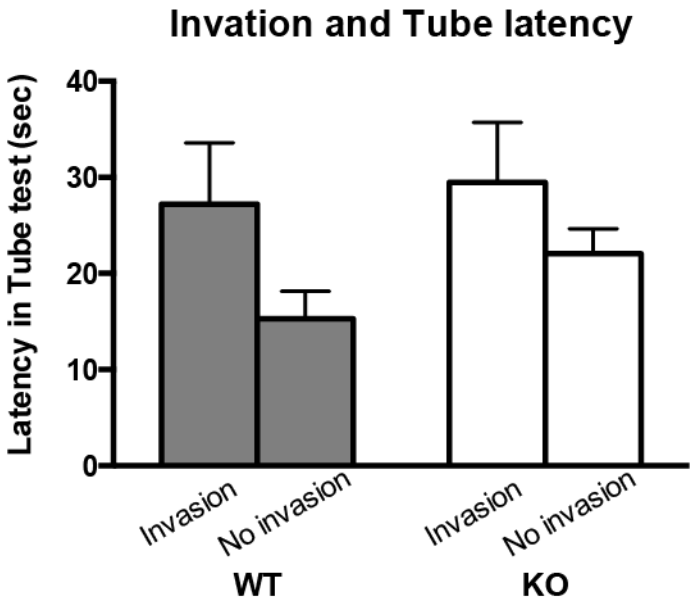

Figure 37. Genotype difference and difference between trials with or without invasion in the latency of loser ejection in the tube test. Both βERKO (KO) and WT (WT) pairs showed shorter latency in the tube test when the trial was with invasion. However, genotype difference was not significant. All data are presented as mean+SEM.

To investigate the relationship between aggressiveness and behaviors in the tube test, "one-sidedness" of aggression in agonistic behavior test was calculated in each trial according to the following equation. One sidedness (%) = (aggression duration by mouse A / total aggression duration of mouse A and B) x 100. Mouse A is a mouse that showed longer aggression duration within a pair. If neither of two mice showed aggression, one-sidedness in that trial was considered as 50%. One-sidedness of aggression was compared between trials with invasion and without invasion in each genotype (Figure 38). In WT pairs, aggression in agonistic behavior test was more one-sided in invasion trials than in no-invasion trials. However, this difference was not observed in KO pairs. Statistical analysis revealed that main effects of genotype and invasion, and interaction of genotype and invasion were significant (genotype: $F_{1,81} = 4.835$, $p < 0.05$, invasion: $F_{1,81} = 5.380$,

$p < 0.05$; genotype x invasion: $F_{1,81} = 6.712$, $p < 0.05$). Post hoc analysis further revealed difference between invasion and no-invasion was significant in WT ($p = 0.001$), but not in KO pairs.

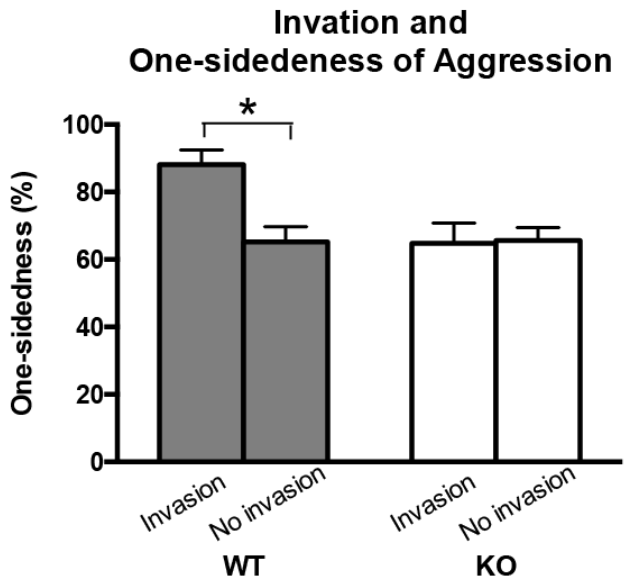

Figure 38. Genotype difference and difference between trials with or without tube test invasion in the one-sidedness of aggressive behavior in agonistic behavior test. WT (WT), but not βERKO (KO) pairs showed lower percentage of one-sidedness of aggression in the agonistic behavior test when subsequent tube test trial was with invasion. All data are presented as mean+SEM.

Taken together, WT mice changed their social interaction by repeated encounter to their partner. In WT pairs, agonistic interactions increased and conflict in the tube test escalated in the latter part of experiment. Moreover, they showed more one-sided aggressive behavior in agonistic behavior test prior to an increase of invasion trial in the tube test, although the “attacker” in agonistic behavior test was not always the winner in tube test (Figure 36). These results suggested that WT mice gradually established dominance hierarchy and their social interaction was consistent between two behavioral

tests.

On the other hand, in KO pairs, agonistic behaviors and loser ejection latency in the tube test were not altered by repeated tests. They showed fewer agonistic interaction and "both" trials in agonistic behavior test. Additionally, there was no relationship of social behaviors in two behavioral tests. These results suggested that deficiency of ERβ gene might affect strategy of establishment of dominance hierarchy.

6.4. Discussion

In this experiment, the role of ERβ in the establishment of inter-male social relationship was examined using βERKO mice. Repeated agonistic behavior tests with a same individual revealed that βERKO mice showed less agonistic interaction and less behavioral change along repeated trials compared to WT mice. A similar trend was observed in the tube test in which social rank of two experimental animals was assessed. In WT pairs, latency to loser ejection became shorter along repeated interaction. It is likely that WT pairs establish their hierarchical relationship through agonistic interaction and conflict in the tube. On the other hand, unchanged social interaction throughout the experiment and a lack of a clear relationship between the results of two behavioral tests in KO pairs suggested tenuous social relationship after repeated interaction.

It is likely that ERβ gene knockout disrupted establishment of social hierarchy between male mice. Agonistic and prosocial behaviors by winners and losers in each trial were additionally analyzed (see Appendix). In WT mice, winners tended to show more aggressive behavior than losers and losers tended to show more fleeing than winners. However, these relationships in the outcome of two behavioral tests were not observed in βERKO mice (Figures A3, A4). In contrast, winner-loser difference in prosocial behaviors was observed in neither genotype (Figures A5, A6). Thus, it is likely that ERβ

is necessary only for establishment of hierarchical relationship in male mice.

Unaltered prosocial behavior including sniffing and approach in βERKO mice was consistent with the previous study using selective ERβ agonist in which subordinate behavior was not affected (Allen et al., 2010). A previous study with social investigation test (same cylinder was used as social recognition test in Experiment 3) for introduction of stimulus animal has reported increased sniffing duration in βERKO males (Handa et al., 2012). Moreover, social instigation, in which pre-exposure to a stimulus male mouse increases following aggressive behavior, was reported in βERKO but not in WT males (Handa et al., 2012). These reports collectively suggested rapid and hyper reaction to same-sex odor in βERKO males, and alteration in aggressive interaction. Shorter latency to loser ejection in the first tube test in βERKO pairs compared to WT pairs also supports the notion of rapid reaction to same-sex individual in βERKO males. The results of social recognition test in βERKO mice suggest that they are able to recognize and memorize their partner. However, after initial reaction to the partner on Day 1, they failed to establish a profound hierarchical relationship with the partner. It is possible that ERβ may be involved in the control of so called “communication skills” in human. Analysis of qualitative alternation in inter-male communication found in βERKO mice is emerging theme in future study.

Taken together, ERβ may be involved in inhibition of hasty reaction to same-sex stimuli and control of well-organized social interaction with dominant behaviors for establishment of hierarchical relationship. On the other hand, the role played by ERβ in the regulation of prosocial interaction and submissive behavior may be minor. In addition, selective ERβ agonist increased dominant and subordinate behaviors in female mice (Allen et al., 2010). It is possible that ERβ may have, at least partially, a common role in establishment of social relationship in male and female mice. It is also intriguing to

investigate the role of ERβ in inter-female social interaction.

-Chapter 7-

General Discussion

7. General Discussion

It is well documented that testosterone plays pivotal roles in male social behaviors. Underlying mechanisms of behavioral regulation by testosterone have been investigated. Relative importance of estrogenic signaling suggested crucial roles of estrogen receptors expressed within the brain. Roles of ERα, which is necessary for turning on of the performance of male sexual and aggressive behavior, has been vigorously investigated. Sano et al. (2013, 2016) revealed brain site- and age- specific regulation of male sexual and aggressive behaviors by ERα using viral mediated site-specific knockdown of ERα gene. On the other hand, the role of ERβ is ambiguous and relatively unknown although ERβ is also distributed within brain sites involved in the regulation of male social behaviors. It has been supposed that behavioral regulation of ERβ is modulatory and fine-tuning because of moderate effect of ERβ gene deletion on the performance of male sexual and aggressive behavior (Ogawa et al., 1999; Nomura et al., 2002). Modulation of social behavior to appropriate level and quality is necessary for animal to live in social group. However, precise neural mechanism and relative importance of behavioral regulation via ERβ is still unclear. In this thesis, I aimed to investigate the role of ERβ in the regulation of male social behaviors by testosterone.

Initially, I conducted site-specific knockdown of ERβ in the MPOA or MeA of pre-pubertal or adult male mice in Experiments 1-4. In these experiment, brain site- and age-specific role of ERβ in the regulation of the performance of sexual and aggressive behavior, and social information processing. I found contribution of pubertal ERβ in the MPOA to full expression of male aggressive behavior. On the other hand, disruption of male-type sexual preference in MeA-βERKD mice suggested that ERβ in the MeA is necessary for male's social information processing related to female's sexual receptivity.

It was further demonstrated that disruption of social information processing about female's receptivity actually affected partner choice of sexual behavior. Thus, ERβ in adult MeA may play an important role in efficient reproduction; i.e. choice of a sexually receptive female.

Furthermore, MeA-βERKD mice showed alteration in investigation of intact male stimulus animal. The precise role of ERβ in inter-male social interaction is not well understood except for the regulation of behaviors in resident-intruder aggression test. In addition to aggressive behavior, it has been suggested that ERβ may have a role in agonistic interaction and social investigation with same-sex individual (Allen et al., 2010; Handa et al., 2012; Tsuda et al., 2014). Thus, in Experiment 5, I intended to examine the role of ERβ in establishment of inter-male social relationship. I found that deletion of ERβ gene disrupted establishment of hierarchical social relationship between two male mice. These results collectively suggested that ERβ may contribute to mate choice and establishment of social relationship which are necessary for animals' survival and reproductive success and that it regulates component of social behaviors differently in each brain site.

7.1. Site-specific regulation of male social behavioral mediated by ERβ

To elucidate neural mechanisms underlying behavioral regulation of ERβ, site- and age- specific roles of ERβ in the MPOA and MeA were investigated. In Experiment 1, it was examined whether ERβ in the MPOA and MeA is necessary for the performance of sexual and aggressive behavior by pre-pubertal site-specific knockdown of ERβ in each targeted brain site. As a result, ERβ knockdown in the MPOA during puberty and adult suppressed aggressive behavior. Unaffected aggressive behavior by MPOA-βERKD only in adulthood in Experiment 2 revealed that pubertal ERβ in the MPOA is involved in the

formation and/or development of neural circuit for facilitation of aggressive behavior. On the other hand, adult knockdown of ERβ in the MeA revealed that activation of ERβ in the MeA in adulthood is necessary for social information processing about female's sexual receptivity. Disrupted sexual preference in MeA-βERKD mice induced disruption of partner choice for subsequent sexual behavior. Moreover, MeA-βERKD males showed subtle alteration of social reactivity to sexually active and same-sex stimulus animal within a cylinder. Thus, ERβ in adult MeA may be also involved in the regulation of social reactivity to same-sex individual.

The roles of ERα in the pubertal and adult MPOA and MeA were also investigated in previous studies (Sano et al., 2013, 2016). Table 2 shows the effect of site-specific ER knockdown in the MPOA and MeA on male social behaviors.

Comparison of the effects of αERKD and βERKD indicated different behavioral regulation of ERα and ERβ in the same brain sites. Co-localization of ERα and ERβ was reported in both MPOA and MeA (Shughrue et al., 1998). How these ERs separately regulate different behavior in different lifetime is emerging question for future study. Profile of neurons expressing ERα and ERβ in these brain sites and intracelluler mechanisms mediating behavioral regulation via ERs have to be elucidated in future.

Moreover, there are other brain sites expressing ERα and/or ERβ. For instance, VMN, BNST, LS, paraventricular nucleus of hypothalamus, and dorsal raphe. The role of ERα in the VMN was also previously examined by site-specific knockdown and it was revealed that ERα in adult VMN is necessary for sexual and aggressive behavior (Table 2, Sano et al., 2013). To elucidate precise mechanisms of estrogenic regulation of neural network for male social behaviors, it is necessary to investigate the role of ERs in other brain regions.

Table 2: Collective results of site-specific knockdown of ERs in the MPOA, MeA, and VMN. Preference FF: Receptive vs Non-receptive female (PTFF), Preference FM: Receptive female vs Intact male (PTFM)

Brain Site	MPOA				MeA				VMN
Receptor Subtype	ERα		ERβ		ERα		ERβ		ERα
Pubertal/Adult	Pubertal	Adult	Pubertal	Adult	Pubertal	Adult	Pubertal	Adult	Adult
Sexual Behavior	↓↓	↓↓	-	-	↓↓	-	-	-	↓↓
Aggressive Behavior	-	-	↓	-	↓↓	-	-	-	↓↓
Preference FF				-				X	
Preference FM				-				-	

7.1.1. MPOA

Site-specific knockdown of ERβ in the MPOA in pre-pubertal period and in adulthood revealed that pubertal ERβ in the MPOA may be involved in the organization of neural network to facilitate male aggressive behavior. Previous studies indicated that testosterone organize the neural network for male social behaviors during pubertal period (Romeo, 2003; Sisk and Foster, 2004; Schulz et al., 2009; Sisk, 2015). Involvement of estrogenic signaling was demonstrated by disruption of sexual and aggressive behavior by site-specific knockdown of ERα in the MeA (Sano et al., 2016). The results in this study initially demonstrated that ERβ also plays a significant role in pubertal organizational action of testosterone.

Although previous studies using βERKO mice (Ogawa et al., 1999; Nomura et al., 2002, 2006) suggested inhibitory role of ERβ in aggressive behavior, the results in Experiment 1 and 2 initially showed that expression of ERβ during pubertal period exerts facilitatory influence on male aggressive behavior. The previous study reported increased aggressive behavior and circulating testosterone level in βERKO male mice during pubertal period, which indicated earlier onset of puberty in βERKO males (Nomura et al., 2002). It was proposed that not only ERα (Lindzey et al., 1998), but also ERβ is involved

in the regulation of gonadal steroid hormone secretion (Temple et al., 2003). Moreover, Nomura et al. (2006) proposed cooperative regulation of aggressive behavior by ERα and ERβ. Potentiated estrogen-inducible aggression in βERKO mice suggested that ERβ may inhibitory regulates aggressive behavior turned on through ERα. This discrepancy might be explained by possible inhibitory activational action in different brain region. ERβ in the MPOA may be necessary for completion of neural network formation for aggressive behavior in pubertal period, whose onset may be regulated by ERβ in the MPOA or other brain regions. After the completion of organization of aggressive behavior neural network in adulthood, ERβ in another brain sites, e.g. LS and dorsal raphe, exert inhibitory regulation on the performance of aggressive behavior. Pubertal organizational action mediated by ERβ was also supported by temporal alteration of ERβ expression. Male-dominant sex difference of ERβ mRNA level in the preoptic area from E15 to P17 (Karolczak and Beyer, 1998) suggested relative importance of ERβ in the developmental period of males. Thus, it is possible that ERβ protein is translated from that mRNA and involved in pubertal organization of MPOA neural network.

In the MPOA, the neural circuit for the regulation of male aggressive behavior wsa indicated in rats and mice. Activation of the caudal MPOA neurons after aggressive encounter was reported previously (Veening et al., 2005). Moreover, reduction of male aggressive behavior by MPOA lesion (Patil and Brid, 2010) suggested the existence of neural circuit that plays facilitatory role on aggression. Recently, Wu et al. (2014) reported that the optogenetic activation of galanin expressing neurons in the MPOA suppressed inter-male aggression. Estrogenic regulation of galanin gene (Marks et al., 1993) and its mediation by ERβ is known (Merchenthaler et al., 2005). Thus, it is speculated that same neurons express both ERβ and galanin regulate inter-male aggression. Although little is known about intracellular mechanisms underlying organizational action mediated by ERβ,

pubertal ERβ might contribute to maintaining activation of galanin expressing neurons at appropriate level and to full expression of aggressive behavior in adulthood.

In the previous studies, Sano et al. (2013, 2016) demonstrated that both of pre-pubertal and adult knockdown of ERα in the MPOA reduced sexual behavior without affecting aggressive behavior. Thus, ERα in the MPOA mediate at least activational action of testosterone although whether it is involved in pubertal organizational action was not clarified. Thus, the role of ERα in the MPOA regulates different aspects of male social behavior from ERβ in same brain site. In the MPOA, it was reported that mRNA of ERβ is co-localized with protein of ERα (Shugrue et al., 1998) although it is unknown whether they are co-expressed in the pubertal MPOA. If pubertal ERα in the MPOA is involved in the organizational action of testosterone, pubertal ERα and ERβ may organize different portion of same neural substrate for male sexual and aggressive behavior respectively. In Experiment 2, βERKD only in adulthood didn't affect the performance of sexual and aggressive behavior and male-type sexual preference. The role of ERβ in adult male MPOA have to be elucidate in future study. Parental behavior is one candidate of the behavior activated by ERβ in adult male MPOA since ERβ in adult female MPOA is reported to have a role in the regulation of maternal aggression and maternal behavior (Nagata et al.; unpublished data).

In the MeA, pubertal ERα is necessary for organization of neural network for sexual and aggressive behavior and full masculinization of the MeA volume, since male mice have larger MeA compared to females (Sano et al., 2016). It is still unknown whether molecular mechanisms underlying organizational action mediated by two ERs are same or different. Underlying molecular mechanisms of ERα- and ERβ- mediated organizational action have to elucidate in future study.

7.1.2. MeA

It was found that ERβ in adult MeA plays an important role in sexual preference of receptive over non-receptive female and preferential copulation to receptive female. Moreover, MeA-βERKD mice showed altered responsibility to intact male stimulus animal in social recognition test. These results suggested that ERβ in adult MeA is involved in social information processing relevant to female's receptivity and that it may modulate inter-male social interaction. However, it was also suggested that the role of ERβ in the MeA might be relatively minor.

Previous study also indicated that ERβ is involved in social information processing relevant to female. In βERKO males, familiarity of female failed to alter risk-taking behavior (Kavaliers et al., 2008). Collectively to the results in this study, it is proposed that ERβ may be responsible for ability to choose female with advantageous features in male's reproduction; i.e. sexually receptive or unfamiliar.

Lesion study using male Syrian hamster proposed that information about sexual attractivity of stimulus animal (odor) is sent from the MeA to MPOA via BNST (Been and Petrulis, 2012). However, lesion of the BNST or MeA-BNST pathway didn't affect the performance of sexual behavior. Thus, it can be suggested that ERβ may be necessary for activation of the MeA-BNST pathway. Notion that different neuronal group in the MeA regulate social and non-social behavior respectively (Hong et al., 2014) suggested complicated neuronal mechanisms within the MeA. In future study, it is necessary to investigate neuronal type and projection site of ERβ expressing neuron.

MeA-βERKD mice showed subtle alteration of social investigation to stimulus male mice in the former half of the social recognition test. As described in discussion of Chapter 5, this phenotype is consistent with that of βERKO male mice. The role of ERβ in the MeA and possible other brain regions in social behavior toward male conspecifics

is discussed in 7.2.2. since the role of ERβ in the MeA might be partial.

Both pre-pubertal and adult βERKD in the MeA didn't affect the performance of sexual and aggressive behavior in adulthood. Previous studies suggested that activational action of estradiol in the MeA, possibly mediated by ERs, regulates male sexual and aggressive behaviors. Lesion of the MeA was reported to affect both sexual and aggressive behaviors (Vochteloo and Koolhaas, 1987; Kondo, 1992; Newman, 1999). Moreover, infusion of estradiol into the MeA of gonadectomized male restored sexual behavior (Wood, 1996). However, either αERKD or βERKD failed to disrupt sexual and aggressive behaviors in male mice. This discrepancy might be explained by the hypothesis that ERα and ERβ in the MeA cooperatively contribute to the regulation of male sexual and aggressive behaviors. Moreover, if ERα and ERβ in other brain regions can be activated, existence of single type of ER in the MeA may be sufficient for the performance of these behaviors. Site-specific infusion of agonists of ERα or ERβ into the MeA of gonadectomized and dihydrotestosterone implanted male rats indicated that both ERα and β are involved in full expression sexual behavior (Russell et al., 2012). Actually, co-localization of ERα protein and ERβ mRNA was also reported in the MeA (Shughrue et al., 1998). Neuronal mechanisms underlying possible cooperative behavioral regulation by ERα and β should be investigated in in future study.

As described in 7.1., effects of site-specific knockdown of ERα and ERβ were different role in the MeA. Neural network organized by ERα in pubertal period is necessary for the performance of sexual and aggressive behavior. Projection of MeA neuron to the MPOA (Been and Petrulis, 2012) and its regulation of dopamine release within the MPOA, which is necessary for the performance of sexual behavior (Dominguez and Hull, 2001). Thus, in contrast to ERβ in pubertal and adult MeA, which plays minor role in the performance of sexual and aggressive behavior, ERα may be

involved in the organization of MeA-MPOA pathway. This study revealed that ERβ in the MeA regulates social information processing at least activationally. Roles of pubertal ERβ in testosterone's organizational action in the MeA remain to be elucidated.

On the other hand, whether the roles of ERα and ERβ in social information processing are same or different is still unclear. In female rats, site-specific knockdown of ERα in the MeA disrupted social recognition (Spiteri et al., 2010). Moreover, αERKD in the MeA disrupted partner preference between novel and familiar female in prairie voles (Cushing et al., 2008). Thus, ERα in the MeA may also be involved in social information processing relevant to female choice. Additionally, Sano et al. (2013) αERKD in adult MeA induced increase of sexual behavior toward intact male mouse during aggressive behavior test. On the other hand, MeA-βERKD group in this study showed unaltered few number of sexual behavior in aggressive behavior test (Figure A7) and unaltered sexual behavior toward non-receptive female. Taken together, ERα and ERβ in the MeA may play basically different role during pubertal period, and their roles can partially overlap in adulthood.

7.2. Contribution of ERβ to the establishment of inter-male social relationship

In this study, I examined the roles of ERβ in social behavior of male mice. As discussed in 7.1., it was revealed that ERβ in the MPOA and MeA mediates the organizational and/or activational action in brain site- and behavior- specific manner.

Pre-pubertal site-specific knockdown of ERβ in MPOA revealed that pubertal ERβ may mediate organizational action of testosterone for aggressive behavior in adulthood. In addition to social information processing about female's receptivity, ERβ in the MeA might be involved in the regulation of reactivity to intact male stimuli. It was suggested that ERβ-mediated activational action is involved in the regulation of inter-male social interaction at least partially. Through analysis of agonistic behavior in βERKO mice

further indicated a role of ERβ in the establishment of inter-male social relationship.

Social information about opponent male animal is conveyed from the MeA to various brain sites including the VMN, MPOA, LS and BNST. Previously, it was suggested that GABAergic neurons in the MeA promote social behavior (Hong et al., 2014). Although profile of ERβ is still unclear, modulation of activation of GABAergic neuron by ERβ can contribute establishment of inter-male social relationship. Additionally, activation of ERα-expressing neurons in the ventrolateral part of the VMN also regulates social interaction to other animal. Lee et al. (2014) revealed these neurons scalably control male's social behavior from sniffing to aggressive behavior. These neuronal group can also be regulated by the ERβ-expressing neurons in the MeA.

Moreover, social information from the MeA is conveyed to the MPOA (Been and Petrulis, 2012). The neural network in the MPOA for aggressive behavior may be formed and/or developed through ERβ-mediated organizational action of testosterone in pubertal period (see Chapters 3 and 4). Although it is not known whether this MPOA neural substrate for aggressive behavior also control non-aggressive social interaction, MPOA is another candidate for the target of ERβ-mediated regulation of inter-male social interaction. As discussed above, neural network including galanin-expressing neurons is possibly organized via ERβ. Since ERβ modulates expression of galanin gene (Merchenthaler et al., 2005), a neuropeptide galanin possibly regulate male's behavior at the downstream of ERβ. I previously analyzed behaviors of male and female galanin-overexpressing transgenic (Gal-OE) mice (Crawley et al., 2002) in social recognition test. Although both sex of animals showed ability of social recognition, female, but not male Gal-OE mice showed increased overall SI duration (Figure A8). On the other hand, male Gal-OE mice showed altered activity; i.e. increased duration of horizontal activity (Figure A9) and that number of vertical activity (standing up; Figure A10). These behavioral

alterations in social situation in Gal-OE mice suggested that galanin can be one of the target molecules for behavioral regulation mediated by ERβ.

Alternatively, neural network for anxiety-related behavior is another candidate to be regulated by ERβ and modulate inter-male social interaction. Previous study using selective ERβ agonist revealed suppression of social anxiety via ERβ (Walf and Frye, 2007). Increased social anxiety in βERKO mice (Walf et al., 2008) may contribute decreased agonistic interaction in agonistic behavior test and tenuous social interaction in the latter half of the Experiment 5. In addition to the MeA, ERβ in dorsal raphe and paraventricular nucleus, which are nuclei originis of serotonin and oxytocin, may contribute the regulation of inter-male social interaction. In Experiment 3, MeA-βERKD mice didn't show any difference in total SI duration toward XM and IM compared to MeA-Cont mice although they showed alteration in SI duration toward one gonadally intact male stimulus mouse. Collectively with the ability of discrimination between gonadectomized and intact male in MeA-βERKD, it is possible that inter-male interaction may be regulated by ERβ only when the opponent is a sexually active male. This hypothesis proposes biological significance of the regulation of inter-male social interaction by ERβ since sexually active but not gonadectomized male can be a rival of male's reproduction.

Taken together, ERβ might regulate male social behavior toward male from multiple aspect; pubertal organizational action in the MPOA of neural circuit for aggressive behavior and activation action in the MeA and possible other brain sites on social reactivity. Moreover, ERβ regulates establishment of appropriate inter-male social relationship through modulation of multiple behavioral components including the performance of aggressive behavior, social investigation, and social anxiety.

In addition, testosterone's regulation of social interaction, possibly mediated by ERβ

can change dependently on situation or environment surrounding an animal. Previously, it is demonstrated that estrogenic signaling was necessary for scent marking, territorial behavior observed in male mice (Kimura and Hagiwara, 1985). Recently, it is also reported that scent marking behavior was altered by testosterone in short-term and that this rapid behavioral regulation was observed only in subordinate individuals (Fuxjager et al., 2015). Further investigation of situation-dependent behavioral regulation by ERβ is necessary.

7.3. Future directions

7.3.1. Identification of type of ERβ-expressing neurons

In this study, I examined the effect of site-specific knockdown or systemic knockout of ERβ on male social behavior. However, neuronal type and profile of the neurons with ERβ expression are still unclear. Moreover, developmental change of ERβ expression within the brain is also unknown. To investigate the profile of ERβ expressing neurons, transgenic mice for visualization of ERβ expression is being developed. Using visualization techniques including immunohistochemistry, profile of ERβ expressing neurons, i.e. GABAergic or glutamatergic, co-localization with other molecules including galanin can be investigated. It is also necessary to investigate whether they are projection neurons or interneuron. If they are projection neuron, projection site can be elucidated using neuronal tracing technique. Moreover, cell-type specific modification of ERβ-expressing neurons, such as optogenetic activation or inhibition can further demonstrated critical roles of ERβ expressing neurons in each expression site in the regulation of male social behaviors.

7.3.2. Investigation of role of pubertal ERβ in social information processing in the MeA and MPOA

In this study, behavioral tests for the assessment of social information processing were conducted only in groups with adult βERKD. In the MPOA, adult βERKD didn't affect male-type sexual preference. Lesion study indicated that not only the MeA, but also the MPOA is highly implicated in male-type sexual preference (Dhungel et al., 2011). It is possible that ERβ in pubertal period play a role in the formation and/or development of neural network for male's social information processing necessary for sexual preference. The MPOA is known to receive innervation of dopamine neurons and essential for sexual motivation of male (Hull et al., 1997). Behavioral test paradigms to distinguish suppressed sexual motivation and disturbed social information processing have to be constructed in case pre-pubertal βERKD in the MPOA disrupts male-type sexual preference. Moreover, role of pubertal ERβ in the MeA is also unclear. Site-specific injection of selective ERβ agonist into the MeA of aromatase knockout mice is a possible experimental plan for identification of the role of pubertal ERβ in social information processing since pre-pubertal βERKD suppress not only pubertal but also adult ERβ expression.

7.3.3. Investigation of precise role of ERβ in establishment of social relationship

In this study, it has been elucidated that deletion of ERβ gene altered establishment of inter-male social relationship and that ERβ in the MeA may be involved in the regulation of reactivity to social stimuli. However, it remains to be elucidated whether βERKO males can establish social relationship after continuous co-habitation. It is known that male mice alter their social behavior toward unfamiliar individual from that toward

cage mates (Winslow and Camacho, 1995). If βERKO males are not able to establish relationship with their cage mates, this behavioral difference may be small compared to WT mice. Through analysis of agonistic interaction in longer duration such as 24 h cohabitation might also provide additional evidence of altered strategy of inter-male social interaction in βERKO male mice. Furthermore, underlying neural mechanism of the regulation of inter-male social interaction by ERβ has to be elucidated using site-specific knockdown in ERβ-expressing brain sites including dorsal raphe and paraventricular nucleus of the hypothalamus.

7.4. Conclusion

The present study provided evidence suggesting pubertal organizational action of ERβ in the MPOA for formation and/or development of neural network for male aggressive behavior. Moreover, it was also revealed that ERβ in the MeA in adulthood might be involved in the information processing about female receptivity and this information processing plays a significant role in actual choice of the partner of sexual behavior. Moreover, analysis of repeated inter-male interaction in βERKO mice suggested importance of ERβ in the regulation of social relationship establishment. Additionally, ERβ in the MeA may be partially involved in the regulation of inter-male social interaction since reactivity to male social stimuli was altered by βERKD in the MeA. It is suggested that ERβ in the MPOA and MeA are involved in the regulation of male social behaviors with brain site-, age-, and behavior-specific manners. In addition, it is further suggested that behavioral regulation mediated by ERβ can contribute mate choice and social relationship, which are essential for reproductive success of male mice, with a certain impact.

Taken together, it is suggested that ERβ regulates multiple aspects of male social

behaviors. 1) Formation and/or development of neural network in pubertal period in the MPOA. 2) Activation of neural network for social information processing relevant to female's receptivity and for the regulation of social reactivity in the MeA. 3) Establishment of appropriate social relationship between males.

-Appendix-

List of Abbreviations:

AAV	Adeno-Associated Virus
αERKO	Estrogen Receptor α Knockout
AGG	Aggressive Behavior test
AHA	Anterior Hypothalamic Area
AOS	Accessory Olfactory Bulb
ANOVA	Analysis of Variance
AR	Androgen Receptor
AromKO	Aromatase Knockout
βERKD	Estrogen Receptor β Knockdown
βERKO	Estrogen Receptor β Knockout
BNST	Bed Nucleus of the Stria Terminalis
BSA	Bovine Serum Albumin
DAB	3,3'-diaminobenzidine
DHT	Dihydrotestosterone
ER	Estrogen Receptor
ERα	Estrogen Receptor α
ERβ	Estrogen Receptor β
FSH	Follicle Stimulating Hormone
GFP	Green Fluorescent Protein
GnRH	Gonadotropin Releasing Hormone
h	hour
IM	Intact Male
LH	Luteinizing Hormone
LS	Lateral Septum
LUC	Luciferase
MeA	Medial Amygdala
min	minute
MOS	Main Olfactory Bulb
MPOA	Medial Preoptic Area
OVX	Ovariectomize
PAG	Periaqueductal Gray
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PBS-X	Phosphate Buffered Saline with TritonX-100
PND	Postnatal Day
PP	Pre-Pubertal
PTFF	Preference Test with Receptive vs Non-receptive Female
PTFM	Preference Test with Receptive Female vs Intact Male

PTMM	Preference Test with Gonadectomized vs Intact Male
PTXFIM	Preference Test with Non-receptive Female vs Intact Male
RF	Receptive Female
RT	Room Temperature
SEM	Standard Error of the Means
SEX	Sexual Behavior Test
shRNA	small hairpin RNA
SI	Social Investigation
SR	Social Recognition
TBS	Tris Buffered Saline
Tris-HCl	Tris Hydrochloride
VMN	Ventromedial Nucleus of the Hypothalamus
wks	weeks
WT	Wild-type
XF	Non-receptive Female
XM	Gonadectomized Male

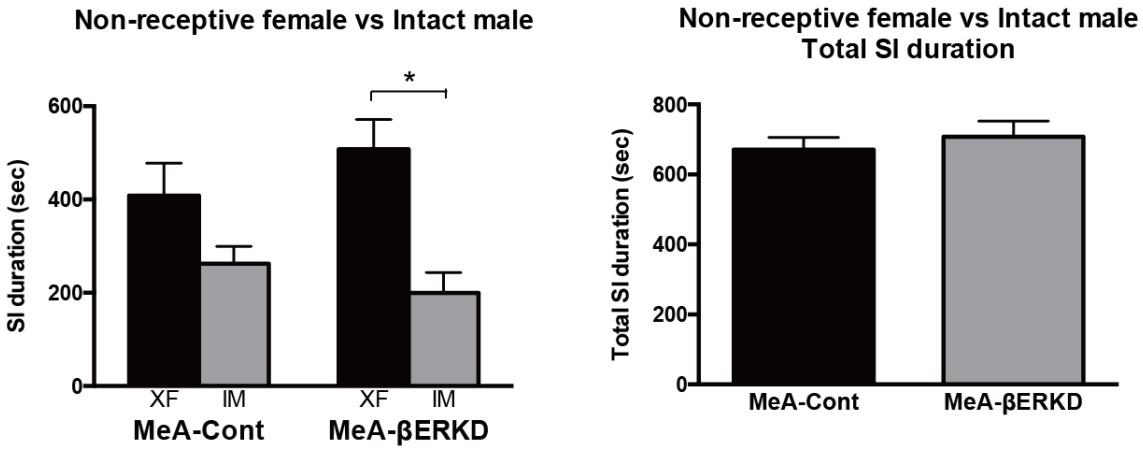

Figure A1 (Chapter 5): Effects of ERβ knockdown in adult MeA on male sexual preference with non-receptive female (XF) and intact male (IM). In the PTXFIM, OVX C57BL/6J female without hormonal priming (XF) and a gonadally intact C57BL/6J male (IM) mouse were used. Testing apparatus and procedures were same as described in General Methods (See 2.3.3.1.) Statistical analysis using paired t-test revealed that MeA-βERKD group showed significantly longer SI duration toward XF (left panel) than toward IM ($t_6 = 3.116$, $p < 0.05$). Although not statistically significant, MeA-Cont group also tended to show longer SI duration toward XF ($t_3 = 1.387$, *n.s.*). Total SI duration of two stimulus animals did not differ between MeA-Cont and MPOA-βERKD groups (right panel; $t_9 = 0.554$, *n.s.;* unpaired t-test). *$p < 0.05$. Data are presented as mean+SEM.

Figure A2 (Chapter 5): Effects of ERβ knockdown in adult MeA on sexual behavior toward non-receptive female. There was no difference between the MeA-Cont and MeA-βERKD groups in either number of mounts (top left panel; $t_9 = 0.840$, *n.s.*), intromissions (top right panel; $t_9 = 0.218$, *n.s.*), or latency to first mount (bottom panel; $t_9 = 0.077$, *n.s.*). Data are presented as mean+SEM.

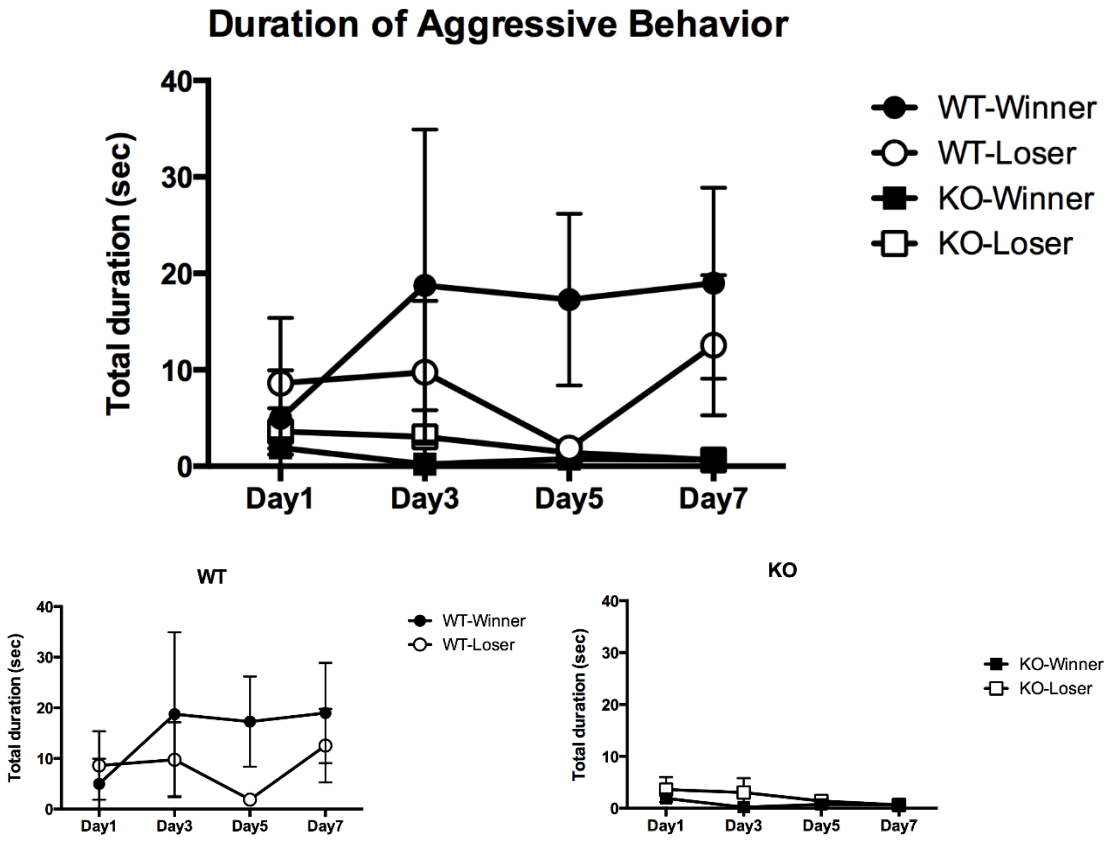

Figure A3 (Chapter 6): Genotype difference in aggressive behavior in agonistic behavior test by winner and loser in the tube test. Because of winner change, winners and losers in each experimental day were different mice. Overall WT-winner tended to show longest duration of aggressive behavior from Day 3 to Day 7 (top panel). Winner-loser comparison revealed that winner tended to be more aggressive than loser in WT pair (bottom left panel). However, winner-loser difference in βERKO pairs was not pronounced (bottom right panel). Data are presented as mean±SEM.

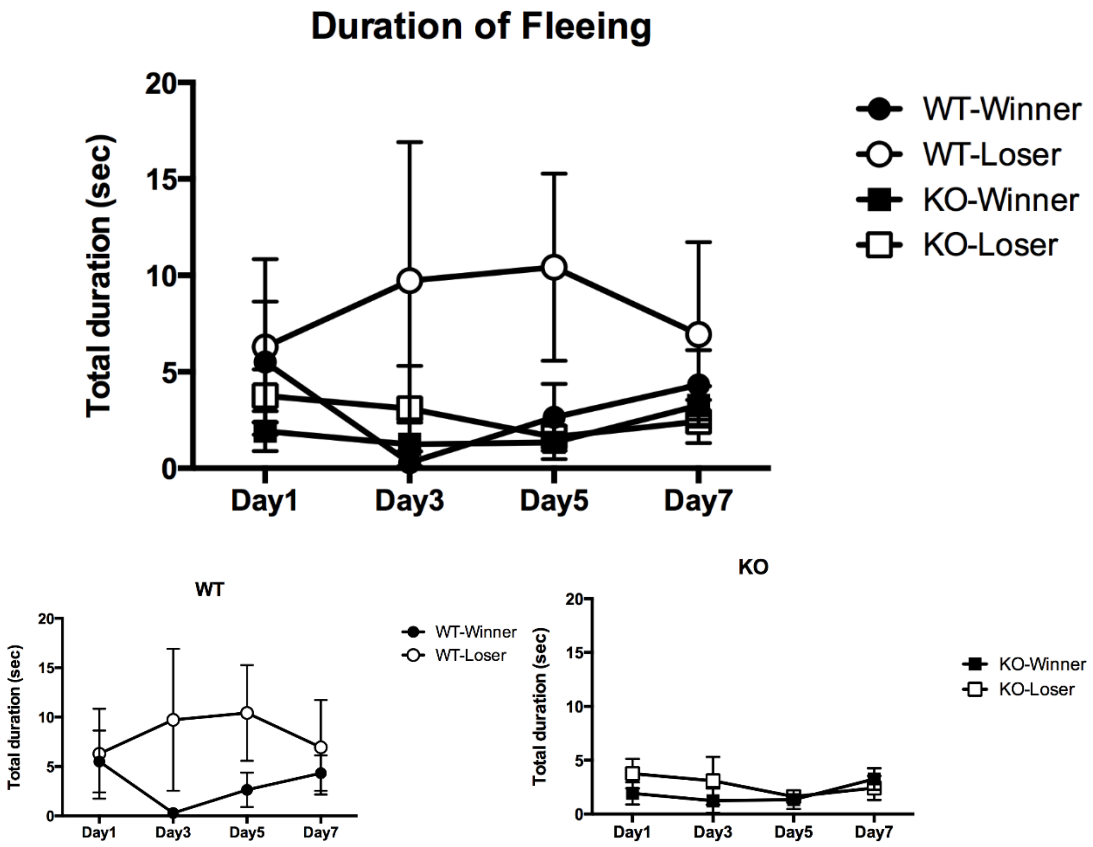

Figure A4 (Chapter 6): Genotype difference in fleeing in agonistic behavior test by winner and loser in the tube test. Because of winner change, winners and losers in each experimental day were different mice. Overall WT-winner tended to show longest duration of fleeing from Day 3 to Day 7 (top panel). Winner-loser comparison revealed that loser tended to be more fleeing than winner in WT pair (bottom left panel). However, winner-loser difference in βERKO pairs was not pronounced (bottom right panel). Data are presented as mean±SEM.

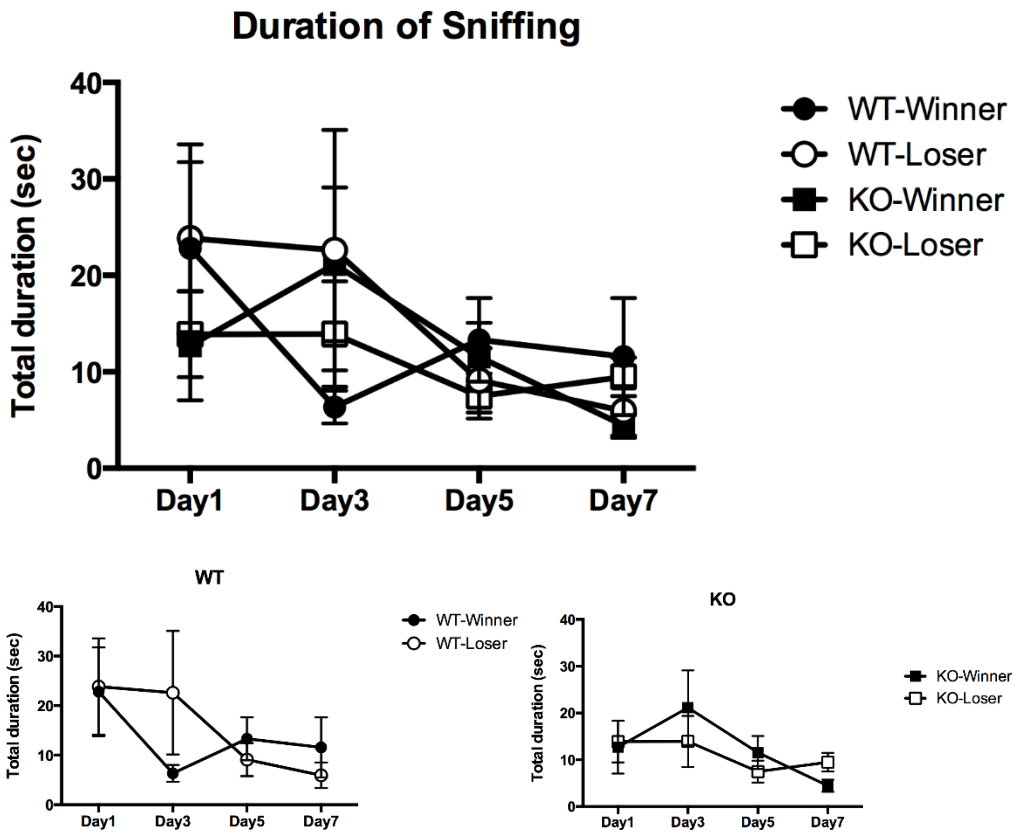

Figure A5 (Chapter 6): Genotype difference in sniffing in agonistic behavior test by winner and loser in the tube test. Because of winner change, winners and losers in each experimental day were different mice. There was no genotype difference in duration of sniffing (top panel). Winner-loser comparison revealed that overall winner-loser differences in WT (bottom left panel) βERKO pairs (bottom right panel) were not pronounced although losers tended to sniff his partner longer on Day 3 in WT pairs and on Day 7 in βERKO pairs. Data are presented as mean±SEM.

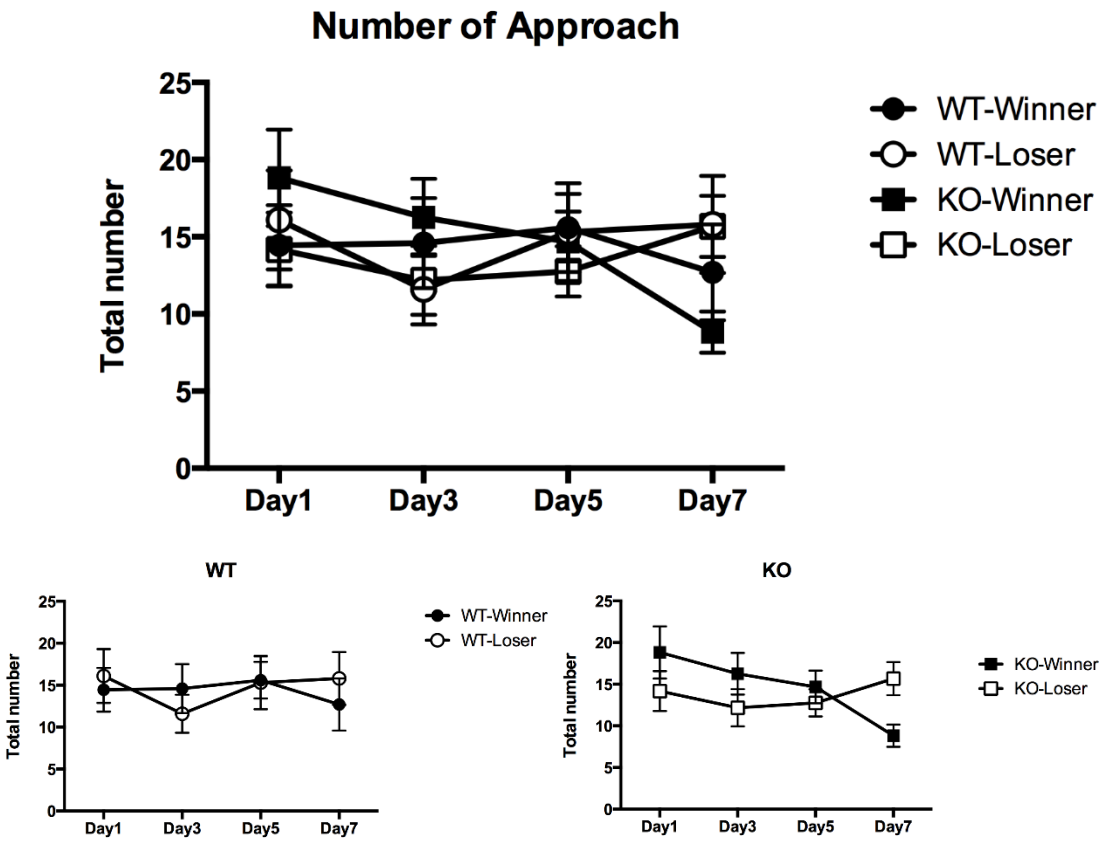

Figure A6 (Chapter 6): Genotype difference in approach in agonistic behavior test by winner and loser in the tube test. Because of winner change, winners and losers in each experimental day were different mice. There was no genotype difference in duration of sniffing (top panel). Winner-loser comparison revealed that overall winner-loser differences in WT (bottom left panel) βERKO pairs (bottom right panel) were not pronounced although losers tended to approach his partner longer on Day 7 in βERKO pairs. Data are presented as mean±SEM.

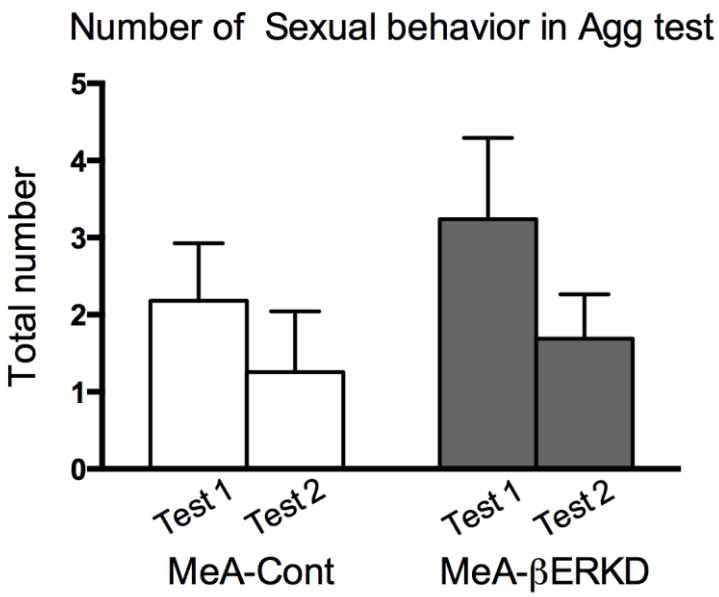

Figure A7 (Chapter 7): Effects of ERβ knockdown in adult MeA on sexual behavior toward OBX male in aggressive behavior test in Experiment 3. There was no difference between the MeA-Cont and MeA-βERKD groups in number of sexual behavior (treatment: $F_{1,25} = 0.141$, *n.s.*; test: $F_{1,25} = 2.304$, *n.s.*; treatment x test: $F_{1,25} = 0.141$, *n.s.*). Data are presented as mean+SEM.

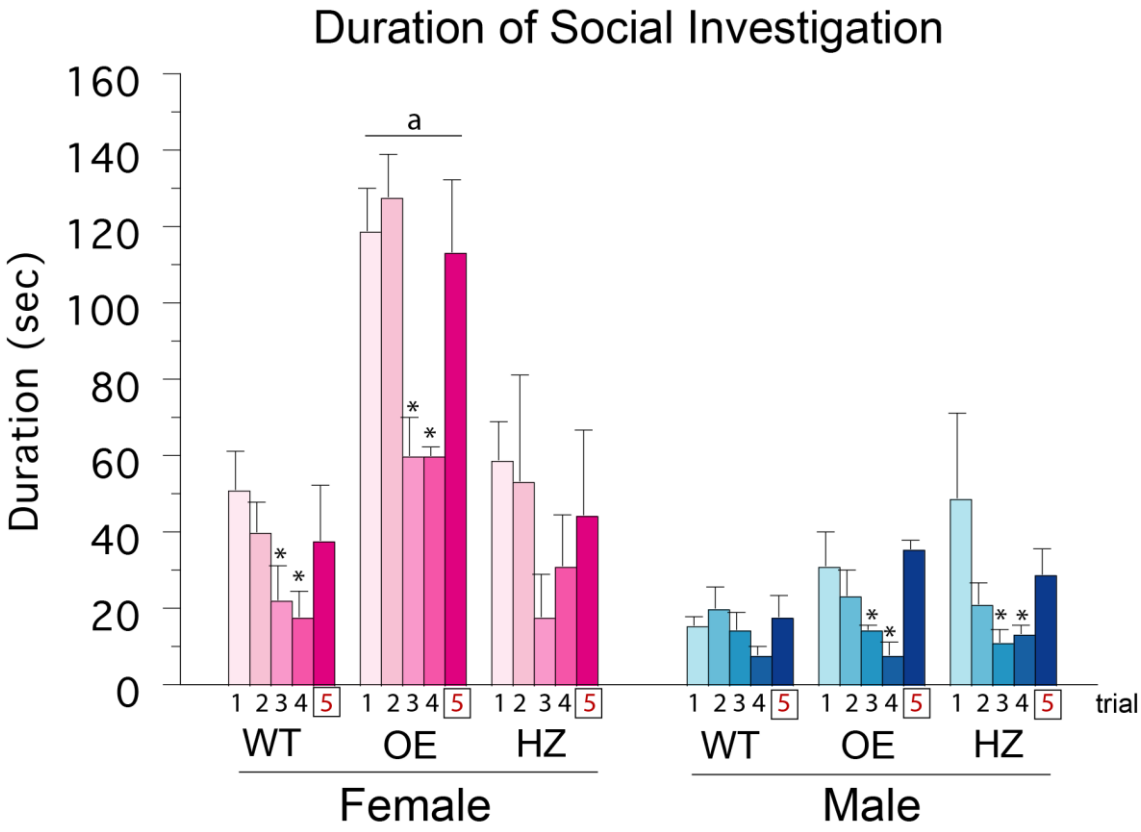

Figure A8 (Chapter 7): Genotype and sex difference in change of SI duration in social recognition test. Experimental animals were a total of 23 adult (20-30 weeks of age) Gal-OE mice (Karolinska Institute line; Female over-expression (OE): n=3, heterozygous (HZ): 4, wild type (WT): n=7; Male OE: n=3, HZ: n=3, WT: n=3). Social recognition tests consisted of five trials of four min duration. Initial four trials were with same stimulus mouse and a novel stimulus mouse was introduced in the trial 5. Test was conducted in home cage of experimental animal using SIOT1 cylinder. In female, OE mice showed longer overall SI duration compared to WT mice ($p < 0.001$). In both sexes, OE mice didn't show disruption in social recognition since SI duration in trial 3 and 4 was significantly shorter than that in trial 1 of the same group ($p < 0.05$). a: $p < 0.001$ vs WT of the same sex, *: $p < 0.05$ vs trial 1 of the same group. Data are presented as mean+SEM.

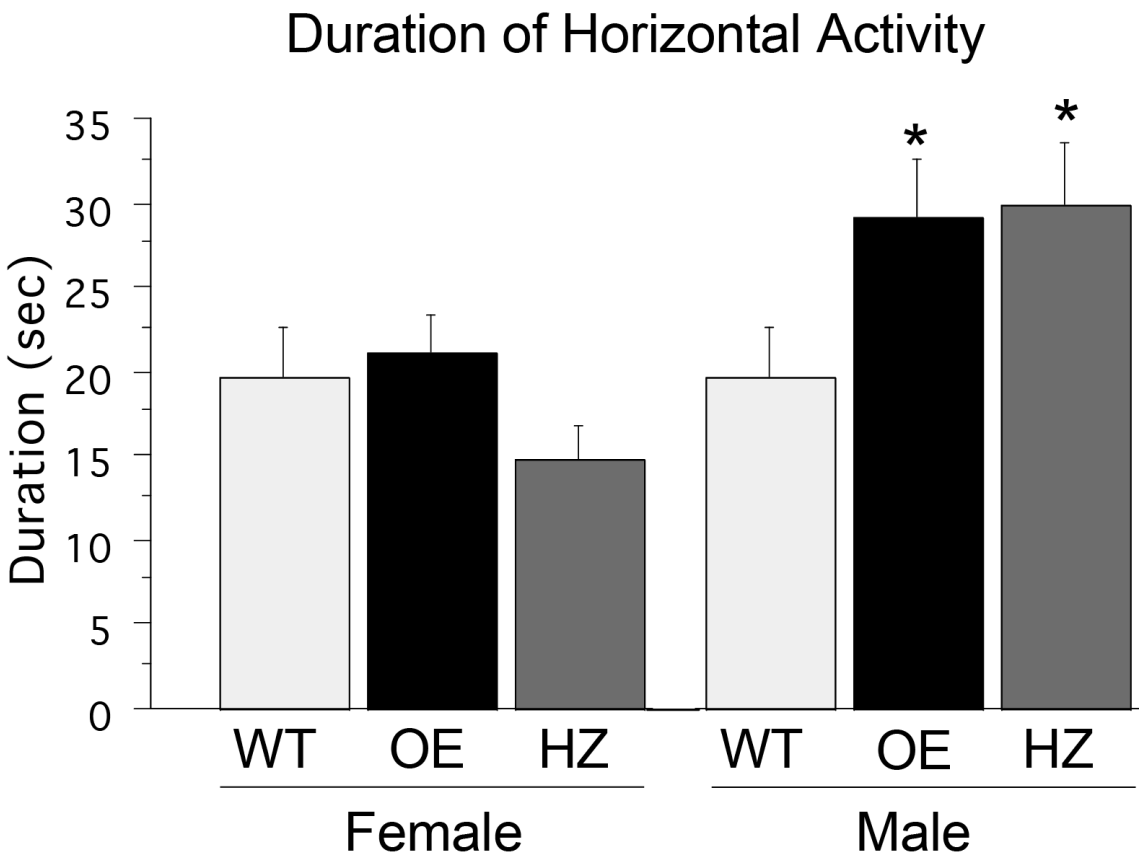

Figure A9 (Chapter 7): Genotype and sex difference in average duration of horizontal activity in social recognition test. Horizontal activity duration was defined as duration of walking or running without SI. In male, OE mice showed significantly longer horizontal activity duration compared to WT mice ($p < 0.05$). *: $p < 0.05$ vs WT of the same sex. Data are presented as mean+SEM.

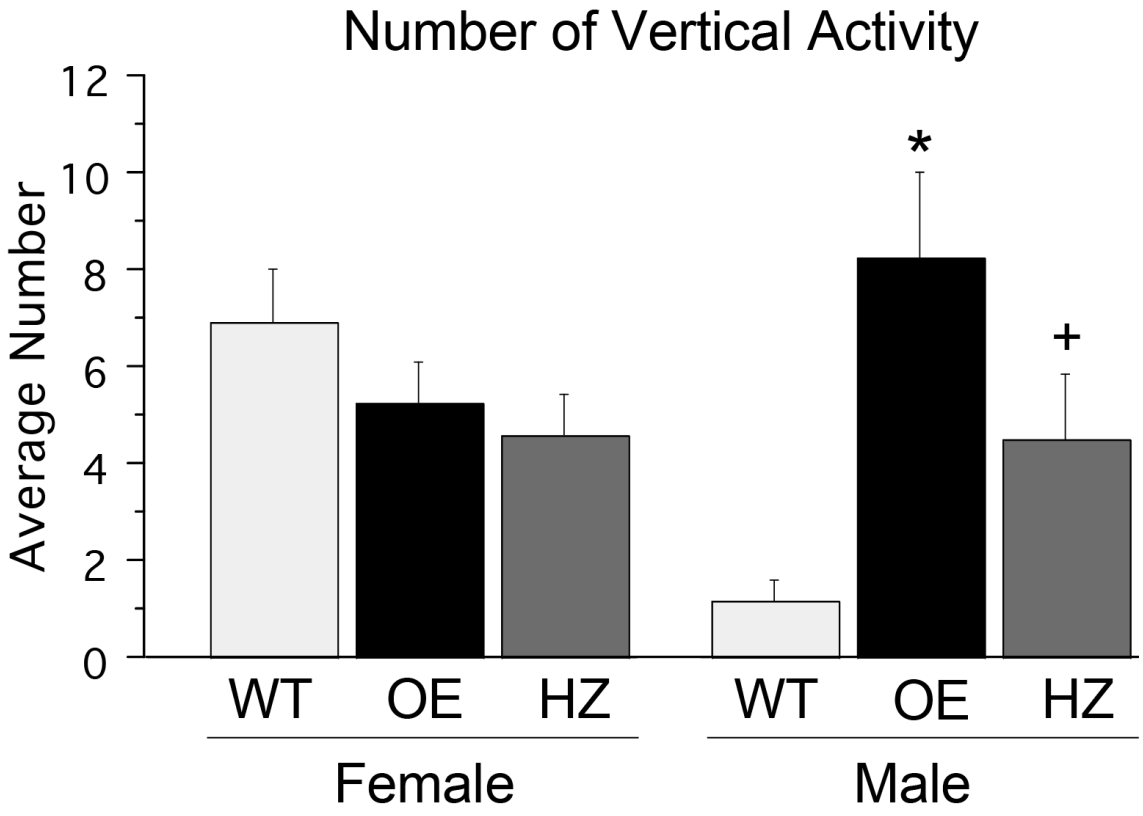

Figure A10 (Chapter 7): Genotype and sex difference in average number of vertical activity in social recognition test. Vertical activity number was defined as number of leaning to the cage wall or rearing (standing up) without SI. In male, OE mice showed significantly longer vertical activity duration compared to WT mice ($p < 0.05$). *: $p < 0.05$ vs WT of the same sex. +: $p < 0.10$ vs WT of the same sex. Data are presented as mean+SEM.

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