Evolutionary studies of sex-related genes in the mouse brain

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Abstract

The brains of most vertebrates are known to exhibit hormonal, chemical and anatomical differences between males and females. However, molecular studies of sexual dimorphism in the vertebrate brains have not been well conducted. Moreover, the evolutionary process of sexual dimorphism in the vertebrate brains is almost unknown. Thus, it is of particular importance to identify the sexually dimorphic genes in the brains and to study the evolutionary process of those genes. In this thesis, I first studied the sexually dimorphic genes expressed in the mouse brain and investigated the evolutionary emergence time of those genes in order to reveal the evolutionary process of sexually dimorphic genes.

In Chapter 1, I described the outline of the present study, making special emphasis on the importance of evolutionary studies for sexually dimorphic genes in the vertebrate brains.

In Chapter 2, with the aim of elucidating the evolutionary process of sexual dimorphism in the brain at the molecular level, I conducted genomic comparisons of a set of genes expressed in a sexually different manner in the mouse brain with all genes derived from 26 eukaryotic species whose complete genome sequences are available. In practice, first, I collected seventeen protein-coding genes whose levels of mRNA expression in the brain differed between male and female mice according to the currently available microarray data. I then designated these genes operationally as "sex-related genes in the mouse brain". Next, I estimated the time when these sex-related genes in the mouse brain emerged in the evolutionary process of eukaryotes by examining the presence or absence of the orthologues in all the eukaryotic species studied here. As a result, I found that ten sex-related genes in the mouse brain emerged after the divergence of urochordates and mammals whereas the other seven sex-related genes in the mouse brain emerged before the divergence of urochordates and

mammals. In particular, five out of the ten sex-related genes in the mouse brain emerged just before the appearance of bony fish which were known to have phenotypic sexual dimorphism in the brain. Interestingly, three of these five sex-related genes that emerged during this period were classified into a functional category of the "protein binding". Moreover, all of these three genes were expected to have the functions that are related to cell-cell communications in the brain according to the gene expression patterns and/or functional information of these genes. These findings suggest that the orthologues of the sex-related genes in the mouse brain having emerged just before the divergence of bony fish might have essential roles such as forming protein-protein interactions in the evolution of the sexual dimorphism in the brain.

In Chapter 3, I focused upon brain region-specificities of sexually dimorphic genes in the mouse and their evolutionary processes. Vertebrate brains generally exhibit anatomical, biochemical and hormonal differences between males and females, and it is easily speculated that the genes manifesting sex-related features in a brain is different among brain regions because distinct brain regions have developed their specific functions at different evolutionary periods. However, little is known whether there are any differences in sex-related features among different brain regions of vertebrates from the evolutionary point of view. To investigate the differences of sexually dimorphic genes among brain regions of the mouse, I conducted comparative analysis of gene expression patterns between male and female mice. Microarray analyses of 18,538 transcripts of mice revealed 41, 44, 11 and 339 sexually dimorphic genes expressed in a preoptic area (POA), a hypothalamus (HY), an olfactory bulb (OB) and a pituitary (PIT), respectively. Furthermore, I found that proportions of brain-region specific dimorphic genes to all of the dimorphic genes in each of brain regions were 87.8% in the POA, 86.4% in the HY, 81.8% in the OB and 98.5% in the PIT, respectively, when genes showing sexually dimorphic expression specifically in a brain region were operationally defined

as "brain region-specific dimorphic genes". Thus, it suggests that the gene expression with sexually dimorphic characters exhibits a regional specificity in the mouse brain. Next, to explain the reason that the differences of gene expression patterns in sexual dimorphic genes among brain regions of the mouse appeared in the evolutionary process, orthologues of the sexually dimorphic genes of each brain region were collected and compared with their evolutionary emergence time. Consequently, I found that the evolutionary emergence time of orthologues of these sexual dimorphic genes for each of the brain regions has varied with brain regions of the mouse. In fact, for the POA and the PIT, the brain region-specific dimorphic genes that emerged during early vertebrates in the evolutionary process were the most abundant among different evolutionary periods. On the other hand, the HY-specific dimorphic genes that emerged just before the divergence of nematodes in the evolutionary process were the most abundant among different evolutionary periods. These results suggest that the differences of gene expression patterns of sexually dimorphic genes among different brain regions might have evolutionarily developed their brain region-specificities.

In Chapter 4, I concluded that the orthologues of the sex-related genes in the mouse brain which emerged just before the divergence of bony fish might have essential roles in the evolution of the sexual dimorphism in the brain by forming protein-protein interactions. Furthermore, I showed that the sexually dimorphic genes among 4 brain regions differ in their evolutionary process and these differences might be important for the development of the brain region-specificities. Finally, I emphasized that the present approach for utilizing a large set of gene expression data and gene gain and loss information is useful to understand the evoltion of sexually dimorphic genes in the brain.

Chapter1: General introduction

1.1 Mechanisms of sexual differentiation in the vertebrate brains

The development of sexual differences in a vertebrate brain is determined primarily through the secretion of steroid hormone such as androgens from the male testis (Breedlove, 1992; Cooke et al., 1998). Once the testis of male is formed it produces androgen, which then reaches the brain and is converted into the estrogen by the enzyme aromatase. This estrogen is responsible for the induction of musculinization in the brain of a male. It has been known that urochordates, close relatives of vertebrates, lack the orthologues of P450 enzyme that is essential for the synthesis of androgens (CYP17) and estrogens (CYP19) (Dehal et al., 2002). Therefore the mechanisms of sexual differentiation in the brain by gonadal hormones likely arose early in the evolution of vertebrates.

1.2 The evolutionary significance of the appearance of sexual dimorphism in the brain of vertebrates

The first goal of my study is to understand sexual differences in the brain from the aspect of evolution. The sex differences in the vertebrate brains contribute to sex-related functions such as sexual behavior and reproduction (Cooke et al., 1998). For examples, vertebrate brains of males and females regulate stereotyped pattern of reproductive behavior during sexual orientation, courtship and copulation (Ch.pilgrim and I. Reisert, 1992). Another well-known example is concerned with the neuroendocrine feed back loop controlling the secretion of gonadotropin such as luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Ch.pilgrim and I. Reisert, 1992). I speculated that the sex-related features in the brain are closely correlated with sex-related genes that emerged either before or after the divergence of

urochordates. In order to elucidate the relationship between the phenotypic sex-related features and sex-related genes in the brain, I estimated the emergence time of a set of sex-related genes in the mouse brain in the course of evolutionary time. The detailed analyses and results are described in chapter 2.

1.3 Sexual dimorphism in the vertebrate brain at the gene expression level

It is expected that the hormonal and chemical differences in the vertebrate brains between males and females should ultimately result in differential gene expression between the sexes, a part of which in turn should control sex-related behavior and physiology in vertebrates. However, despite dramatic behavioral differences between the sexes, there are only rare examples of molecular differences between the sexes (Cooke et al., 1998). So far, it has been considered that sex-related expression differences are primarily limited to genes on the sex chromosome (Rinn et al., 2005). However, Yang et al. (2006) showed that there are significant sex differences in gene expression in the mouse brain by microarray analyses. This analysis have allowed us to investigate the evolution of sex-related features in the mouse brain at the molecular level.

Taking the similar approach as Yang et al., (2006), I also conducted oligoarray of 18,538 transcripts to identify the sexual dimorphic genes expressed in specific brain region of male and female mice. Instead of analyzing the gene expressions of the whole brain tissue from both male and female (as described in Yang et. al., (2006)), I specifically focused on four brain regions: preoptic area (POA), a hypothalamus (HY), an olfactory bulb (OB) and pituitary (PIT), which are thought to be involved in the sexual difference in brain. As I would expect, my approach may increase the sensitivity for detecting any differential gene expressions in the above brain regions between male and female mice.

1.4 The availability of a large scale analysis of gene gain and loss information for evolutionary study

New gene functions are required for organisms to develop new morphological traits and biochemical processes (Burki and Kaessmann, 2004; Long et al., 2003). One way to understand the process of phenotypic acquisition is to study the gene gain and loss events of orthologous genes that are related to the phenotypic traits of organisms in the course of evolution. Previous studies using this "gene gain and loss" analysis have been focused on the evolutionary process of the brain at the molecular level. (Mineta et al., 2003; Noda et al., 2006). For instance, Noda et al. (2006) proposed that the molecular cause of the brain evolution was the addition of new genes which took place most actively just before or at the evolutionary emergence of vertebrates. To date, the evolution of sexual dimorphism in vertebrate brains has only been studied at the morphological and hormone levels and little is known about the evolution of sexual dimorphism in vertebrate brains at the molecular level, with the exception of gonadal hormone-related genes such as estrogen receptors. Therefore, I intended to investigate a set of 17 sex-related genes in the mouse brain in relation with their emergence times in the evolutionary process. As reported in the following chapter, I surveyed all orthologues of these 17 genes in 26 eukaryotic species and counted the number of gene gain and loss events in each lineage. Moreover, I also examined the emergence time of sexually dimorphic genes at different brain regions of mouse, given that the tissue specific-genes are useful to find possible link between the evolution at the molecular and tissue levels (Miyata et al., 1994). These brain region-specific, sexually dimorphic genes were obtained from the microarray analyses that I carried out in chapter 3. These analyses are particularly important to gain an insight into the gain and loss events of sexually dimorphic genes expressed in brain specific-regions and may further provide useful information for the evolutionary study of tissue-specific genes in vertebrates.

1.5 Main findings

In chapter 2, I raised a question of whether the sex-related genes that are expressed in the mouse brain have emerged almost simultaneously with the phenotypic appearance of sexual dimorphism in the brain. To answer the question, I conducted genomic comparisons of a set of genes expressed in a sexually different manner in the mouse brain with all genes from other species of eukaryotes. Consequently, I found that the orthologues of the sex-related genes in the mouse brain that emerged just before the divergence of bony fish might have essential roles in forming protein-protein interactions during the evolution of the sexual dimorphism in the brain.

In chapter 3, I estimated and compared the evolutionary emergence times of sexually dimorphic genes among four brain regions in the mouse. In the first half of the chapter, I conducted comparative analyses of gene expression between males and females in a preoptic area (POA), a hypothalamus (HY), an olfactory bulb (OB) and a pituitary (PIT), by microarray analyses. In the second half, I compared the orthologues of sexually dimorphic genes among the four brain regions upon the evolutionary period when they have emerged. Consequently, I found that the evolutionary process of sexually dimorphic genes differs among the brain regions and these differences might lead to the formation of sex-related features and specificities at each region in the vertebrate brain.

Chapter 2: Evolutionary origin of sex-related genes in the mouse brain

2.1 Introduction

The brains of most vertebrates are said to exhibit phenotypic sex differences between males and females. In particular, the hormonal, chemical and anatomical differences between the two sexes have been intensively studied to date in various species of vertebrates (Cooke et al., 1998). In rats, for example, quantitative differences in cell numbers have been observed in the preoptic nuclei of the preoptic area (Raisman and Field, 1971) and the bed nucleus of the stria terminalis located between the amygdala and hypothalamus (Huton et al., 1998). The physical size of the preoptic nuclei of the preoptic area is also 3-4-fold larger in male rats than in female rats (Gorski et al., 1980). Similarly, the analogous region of the human preoptic area is larger in men than in women (LeVay et al., 1991). Among birds, Nottebohm and Arnold (1979) reported that the song control nuclei in the brain of zebra finches and canaries have 5-6-fold larger volumes in males than in females. Among amphibians, the neurons of *Xenopus laevis* constituting the courtship song neural circuit are sexually dimorphic in their cell numbers and volumes (Simpson et al., 1986). Among fishes, there are sex differences in the vasotocinergic neurons in the brain of the goldfish (Parhar et al., 2001) and medaka (Ohya et al., 2006).

In vertebrate brains, gonadal hormones, particularly estrogen and androgen, play essential roles in masculinizing the nervous system (Cooke et al., 1998). In rats, for example, the estrogen that is aromatized from testicular androgen interacts with estrogen receptor to induce a masculine preoptic area in a brain (Morris et al., 2004). Among birds, the aromatization of testicular androgen into estrogens causes the developing brain of zebra finches

to become masculinized (Gurney and Konishi, 1979). Androgen secretion by males has been shown to masculinize the frog song system (Cooke et al., 1998).

To date, the evolution of sexual dimorphism in vertebrate brains has only been studied at the morphological and hormone levels. In fact, little is known about the evolution of sexual dimorphism in vertebrate brains at the molecular level, with the exception of gonadal hormone-related genes such as estrogen receptor, although molecular evolutionary analyses previously provided novel clues about the evolutionary process in the brain (Mineta et al., 2003; Noda et al., 2006).

Yang et al. (2006) showed that there are sex differences in gene expression in the mouse brain. They identified 17 genes whose levels of mRNA expression differed between male and female mouse brains at a fold change level of more than 1.3. These data have allowed us to investigate the evolution of sexual dimorphism in the mouse brain at the molecular level. In this study, I defined these sexually dimorphic genes expressed in the mouse brain operationally as "sex-related genes in the mouse brain".

To understand the evolution of sexual dimorphism in the mouse brain at the molecular level, I focused on gene gain and loss events because new gene functions may be required for organisms to develop new morphological traits and biochemical processes (Burki and Kaessmann, 2004). In our analyses, I divided the entire process of eukaryotic evolution into the eight periods of time as described in Noda et al. (2006). Designation from (A) to (H) (see Fig. 2.2B) specified the period during which a given sex-related gene in the mouse brain emerged. In particular, I focused on the time when mammals and urochordates diverged since morphological and hormonal sex differences in the brain regulated by sex hormones are found throughout the species that diverged after urochordates (Cooke et al., 1998). In this study, I show that the orthologues of the sex-related genes in the mouse brain that emerged in early

vertebrates might be essential for evolution of the sexual dimorphism in the brain.

2.2 Materials and Methods

2.2.1 Definition of sex-related genes in the mouse brain

To identify the sex-related genes in the mouse brain, I used the dataset of sexually dimorphic genes in the mouse brain compiled by Yang et al. (2006). I chose to use their data because they used a very large set of animals (169 male mice and 165 female mice) to identify sexually dimorphic genes in the brain and the data are therefore credible statistically. Yang et al. (2006) defined the genes exhibiting biologically relevant expression in the mouse brain as "actively-expressed genes" (for details on the methods, refer to Yang et al., 2006). In this study, I defined the genes showing sexually dimorphic expression among these actively-expressed genes as "sex-related genes in the mouse brain". In practice, I used the genes whose mRNA levels showed 1.3-, 1.5- and 2.0-fold differences between male and female mouse brains (Supplementary Table 2.1). The information for all the mouse genes was obtained from Ensemble release 40 at the Wellcome Trust Sanger Institute. The Ensemble Gene IDs of 24,438 mouse protein-coding genes were extracted from the Mus_musculus.NCBIM36.40.pep.all.fa file, which was downloaded from ftp://ftp.ensembl.org/pub/current_mus_musculus/data/fasta/pep/.

2.2.2 Estimating the evolutionary emergence time of sex-related genes in the mouse brain

To understand the evolutionary process of the sex-related genes in the mouse brain, I investigated the proportion of the sex-related genes in the mouse brain that emerged during each "period" of time for a given phylogenetic tree (Fig. 2.2B). I defined each period as an evolutionary time separated by epoch-making branching nodes in eukaryotic evolution as shown by Noda et al. (2006). A total of 8 periods of eukaryotic evolution were designated (A) to (H) as shown in Fig. 2.2B.

To estimate the evolutionary emergence times of the sex-related genes in the mouse brain, I used PhyloPat database v41 (http://www.cmbi.ru.nl/pw/phylopat/41/) (Hulsen et al., 2006), which reveals the presence or absence of protein-coding genes in 26 species of eukaryotes (phylogenetic pattern) (see Supplementary Table 2.1) using the orthologous relationships defined by the phylogenetic trees of these genes. The phylogenetic patterns of 17 sex-related genes in the mouse brain and 24,438 mouse genes were obtained from the PhyloPat database. For each of these phylogenetic patterns, the most primitive species that possessed orthologues of these genes were identified (Supplementary Table 2.1). Next, I estimated the number of genes emerging during each of the 8 evolutionary periods in which the most primitive species existed.

2.2.3 Classification of sex-related genes in the mouse brain into molecular function categories

The molecular functions of the sex-related genes in the mouse brain were assigned according to the Gene Ontology (GO) classification. The classification of the sex-related genes in the mouse brain into molecular function categories of GO was conducted using the web tool Fatigo (http://fatigo.bioinfo.cipf.es/) (Al-Shahrour, 2004).

2.3 Results and Discussion

2.3.1 Emergence of sex-related genes in the brain before and after the divergence of urochordates

As already mentioned, it has been suggested that sexual dimorphism in morphologies and hormones is found in the nervous system throughout vertebrates (Cooke et al., 1998). To clarify the relationship between the emergence of the sex-related genes in the mouse brain and the emergence of anatomical and hormonal sex in the brain during the evolutionary process, I investigated the sex-related genes in the mouse brain that emerged before and after the divergence of urochordates, respectively. As a result, I found that the proportions of the sex-related genes in the mouse brain that emerged after the divergence of urochordates and mammals (evolutionary periods from (E) to (H)) were 58.8%, 75.0% and 60.0% according to three different criteria with sexual differences of more than 1.3-fold, 1.5-fold and 2.0-fold, respectively (Fig. 2.1). On the other hand, 49.7% of all the mouse genes emerged after the divergence of urochordates and mammals (Fig. 2.1). As shown in Fig. 2.1, the sex-related genes in the mouse brain, with criteria of 1.3, 1.5, and 2.0 folds, emerged both before and after the divergence of urochordates and mammals.

Furthermore, I found that the top molecular function category of GO of the sex-related genes in the mouse brain that emerged after the divergence of urochordates is "protein binding" (Table. 2.1). Therefore, the emergence of "protein binding" genes after the divergence of urochordates might contribute to the formation of protein-protein interactions in the evolution of elaborated sex-related features of the vertebrate brains.

It has been shown that estrogen receptor, androgen receptor and progesterone receptor, which play critical roles in sex-related functions such as sexual behavior (Ogawa et al., 1997;

Sato et al., 2004; Schneider et al., 2005), emerged in early vertebrates (Baker, 2003). Tanaka et al. (2006) have suggested that an extraordinary number of genes involved in estrogen and androgen metabolism and C21-steroid hormone metabolism (including progesterone synthesis), which produce the ligands of estrogen receptor, androgen receptor and progesterone receptor, respectively, were also gained in the vertebrate lineage. Taking these studies into consideration, I propose that the emergence of sex-related genes in the brain during vertebrate evolution might be essential for formation of sexual dimorphism in the brain.

2.3.2 Characteristics of sex-related genes in the mouse brain that emerged during each evolutionary period of time

As already mentioned, I defined 8 evolutionary periods to clarify the relationship between the emergence of these 17 sex-related genes in the mouse brain and the evolutionary process of sexual dimorphism in the brain (Fig 2.2B). I found that 6 (approximately 35.3%) of the 17 sex-related genes in the mouse brain emerged before the divergence of yeasts (outgroup) and mammals, namely during period (A) (Figs. 2.2A and B). In addition, this proportion (35.5%) was higher than that of all the mouse genes during the same period (Fig. 2.2A). These findings suggest that the orthologues of these 6 sex-related genes in the mouse brain have already existed in the common ancestor of yeasts and mammals, which have no sexual dimorphism.

Next, I investigated chromosomal locations of the orthologes of these 6 genes that emerged during period (A) to examine whether the sexual dimorphic expressions of these genes are correlated with the sex-chromosomal location or not. Interestingly, I found that 5 of these 6 genes that emerged during period (A) were located on either X or Y chromosome in some mammalian species including mice. This result indicates that these 5 genes manifested the

dimorphic gene expression according to their location on the sex chromosomes rather than acquirement of sex-related regulation of these genes by gonadal hormone in the evolutionary process.

Moreover, I examined the functions of these 6 genes by GO. As shown in Table 2.1, 4 genes of Ddx3y, Ddx3x, Eif2s3x and Jarid1d were classified into "nucleic acid binding" category by GO. At the gene expression level, all of these 6 genes were expressed in most of organs or organ systems (9 or 10 organs or organ systems) in mice according to BodyMap-Xs database (Ogasawara et al., 2007; http://bodymap.jp/). Thus, 6 sex-related genes that emerged in period (A) would have basic functions in a cell of vertebrate brains.

To examine whether there exist sex-related genes in the mouse brain that emerged at almost the same time as the appearance of sexual dimorphism in the brain at the phenotypic level, I investigated the proportion of the sex-related genes in the mouse brain that emerged during period (E). As a result, I found that 29.4% (5 of 17) of sex-related genes in the mouse brain (sexual differences of more than 1.3-fold) emerged during period (E) (Figs. 2.2A and B). Moreover, the proportion of the sex-related genes in the mouse brain that emerged during period (E) was higher than that of all the mouse genes that emerged during the same period (Fig. 2.2A). The same results were obtained in the case of sexual differences of more than 1.5-fold and 2.0-fold (data not shown). Therefore, gene gain events of these 5 sex-related during period (E) might be required to form the sexual dimorphism in the brain in the evolutionary process.

To clarify the characteristics of the sex-related genes in the mouse brain that emerged at almost the same evolutionary period as the appearance of phenotypic sex differences in the brain, I examined the GO "molecular function" categories of these 5 sex-related genes in the mouse brain that emerged during period (E). As a result, I found that 3 out of these 5 sex-related genes in the mouse brain, prolactin (Prl), thyroid-stimulating hormone beta subunit

(Tshb) and an unknown mouse gene (4930488E11Rik) that emerged during period (E) were classified to the "protein binding" category in GO (Table 2.1 and Fig. 2.2A). In particular, Prl and Tshb also belonged to the "receptor binding" function category. I found that the receptors for Prl and Tshb also emerged during period (E), since Prl and Tshb emerged in the same period (data not shown). Thus, it was considered that cell-cell communications by these two peptide hormones and their receptors were evolutionarily generated in this period. In addition, 4930488E11Rik had thymosin beta-4 domain according to Interpro (IPR001152). Thymosin beta-4 is a small polypeptide that has been shown to bind to actin monomers. It has been considered that the genes having this domain are involved in developmental regulation of central nervous systems controlling cell shape in vertebrates (Lugo et al., 1991; Yamamoto et al., 1994; Roth et al., 1999). Therefore, these three sex-related genes that are classified into "protein binding" category would contribute to cell-cell communication in the vertebrate brain.

It has been known that Prl and Tshb are involved in sex-related function in the brain and pituitary in vertebrates. At the gene expression level, Prl and Tshb were expressed only in the brain including pituitary in mice according to BodyMap-Xs database. Thus, these two peptide hormones would be involved in sex-related function dominantly in the brain. As an example of the evolution of the sex-related function in the brain that is specified by the sex-related gene in the brain, I compared the sex-related function of Prl among vertebrates because prolactin was known to be essential for sex-related behavior. In mammals, Prl and its receptor of mice are involved in sex-related behavior such as maternal behavior (Lucas et al,, 1998; Bridges et al., 1985, 1990). Birds also respond to prolactin by an increase in nesting behavior, nest attendance and incubation behavior (Bole-Feysot et al., 1998). In fishes, Prl and its receptor have a role in the regulation of parental behavior (Power et al., 2005). Taken altogether, it is likely that the formation of cell-cell communication such as endocrine systems in

this evolutionary period affected the evolution of the sex-related characters in the vertebrate brain.

Finally, I would like to emphasize that the approach to utilize public gene expression data and gene gain and loss information is useful to correlate the evolution of a given phenotypic feature with a process of molecular evolution. In the future, evolutionary studies of sexual dimorphism in the brain should be conducted focusing on particular brain regions in order to discuss the evolution of sex-related features that are related to a given brain region.

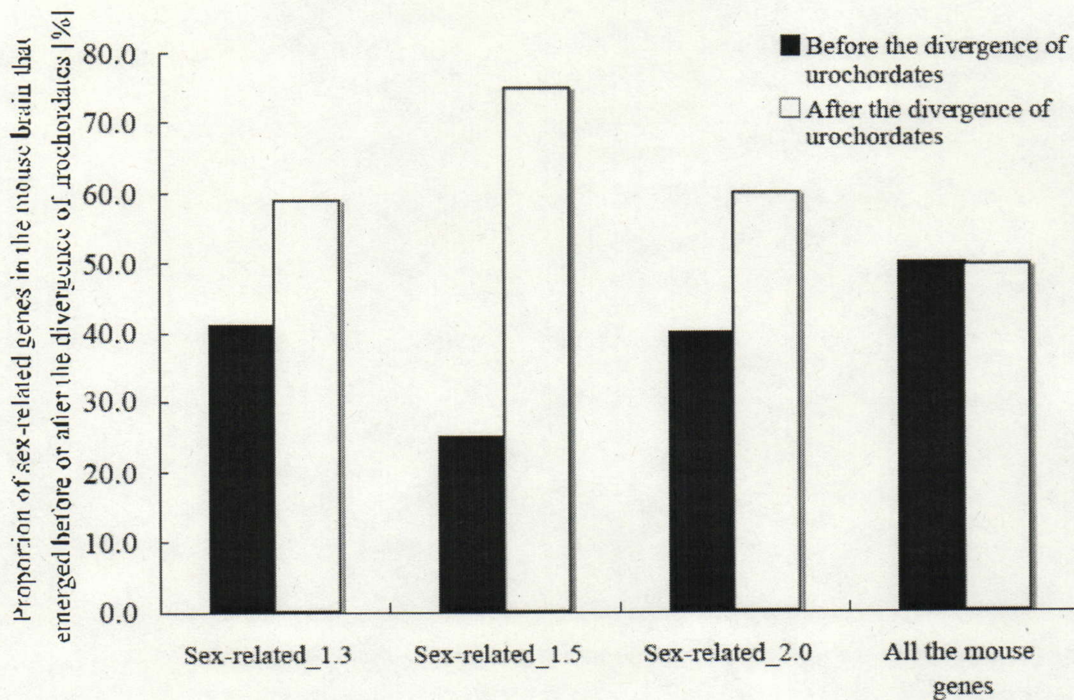

Fig. 2.1 Emergence of sex-related genes in the brain before and after the divergence of urochordates. The proportions of sex-related genes in the mouse brain that emerged before and after the divergence of urochordates. Sex-related_1.3, _1.5 and _2.0 indicate sexual differences of more than 1.3-fold, 1.5-fold and 2.0-fold, respectively. Closed bars indicate the proportion of sex related genes in the mouse brain that emerged before the divergence of urochordates, while open bars indicate the proportion of sex-related genes in the mouse brain that emerged after the divergence of urochordates.

Table 2.1 Molecular function categories of the sex-related genes after and before the divergence of urochordates

After the divergence of urochordates

Molecular function	Number	Gene Symbol
Protein binding	3	Tshb, Prl, 4930488E11Rik
Ion binding	2	S100a8, S100a9
Transferase activity	2	Lrg1, Nalp5
Nucleotide binding	1	Nalp5
Enzyme inhibitor activity	1	Ngp
Unknown	2	BC022960, Camp

Before the divergence of urochordates

Molecular function	Number	Gene Symbol
Nucleic acid binding	4	Ddx3y, Ddx3x, Eif2s3x, Jarid1d
Nucleotide binding	3	Ddx3y, Ddx3x, Eif2s3x
Ion binding	3	Jarid1d, Hccs, Myl1
Hydrolase activity	2	Ddx3y, Ddx3x
Helicase activity	2	Ddx3y, Ddx3x
Lyase activity	1	Hccs
Protein binding	1	Jarid1d
Unknown	1	Utx

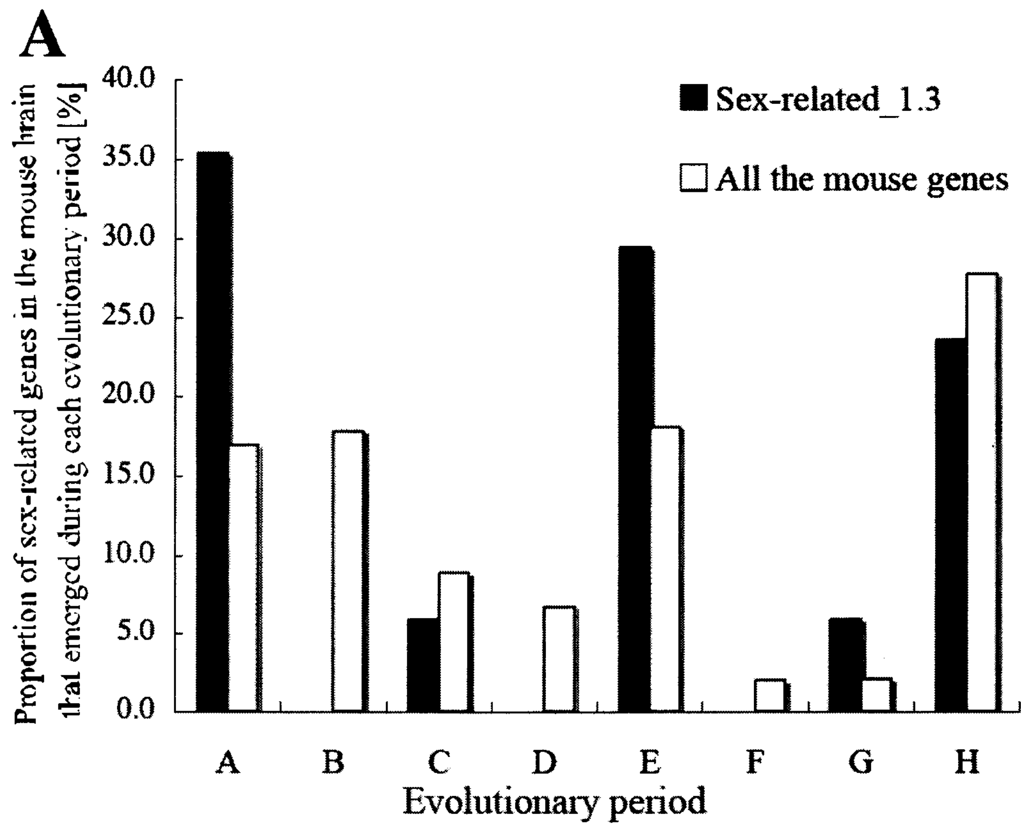

Fig. 2.2 (A) Proportions of sex-related genes in the mouse brain that emerged during each evolutionary period of time. x-axis shows the evolutionary periods which are designated (A) to (H) as shown in Fig 2B.

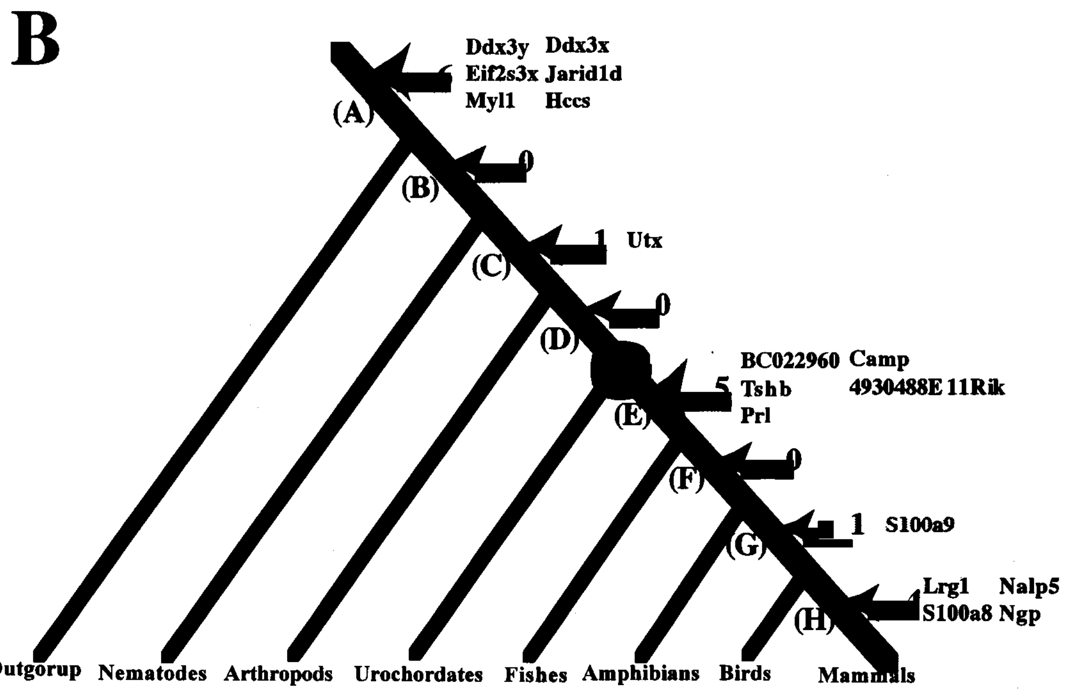

Fig. 2.2 (B) Schematic tree showing the emergence of 17 sex-related genes in the mouse brain during each evolutionary period of time.

Chapter 3: Brain region-specific expression of sexually dimorphic genes in the mouse brain and its evolutionary implication

3.1 Introduction

In general, all vertebrates show sexual dimorphism in the brain at the anatomical and molecular levels, and this is believed to be controlled by gonadal hormones (Breedlove et al., 1992; Cooke et al., 1998). The sexual dimorphism in vertebrate brains is related to the differences in sex-related behavior and reproduction (Pilgrim and Reisert, 1992). For example, the preoptic area (POA) and hypothalamus (HY) are brain regions showing relatively larger sexual dimorphisms than other areas, and are involved in reproduction and sexual behavior in rodents (Breedlove et al., 1992; Cooke et al., 1998; Morris et al., 2004). In birds, Nottebohm and Arnold (1979) reported that the song control nuclei in the brains of zebra finches and canaries, which are related to sexual behavior, have 5-6-fold larger volumes in males than in females. Among amphibians, the neural circuit for the courtship song in *Xenopus laevis* is sexually dimorphic in terms of cell number and volume (Simpson et al., 1986).

Previous evolutionary analyses at the molecular level provided us with novel clues for understanding the evolutionary process in the brain (Mineta et al., 2003; Noda et al., 2006). For instance, Noda et al. (2006) proposed that the molecular cause of brain evolution was the addition of new genes which took place most actively just before or at the evolutionary emergence of vertebrates. However, there has been almost no study that tried to elucidate the evolution of sexual dimorphism in a given brain region. An evolutionary analysis of sexually dimorphic genes in a given brain region would help us to connect the evolution of sex-related

phenotypic features in the brain with the molecular evolution of sexually dimorphic genes, because a given region has a specific function in the brain (Kandel, 2000).

First, I investigated whether there are genes that are expressed in a sexual dimorphic manner, specifically, in the POA, HY, OB or PIT, which are known to be involved in sex-related features at the phenotypic level (Moris et al., 2004). This approach was taken because tissue-specific genes are useful for finding a possible link between evolutionary processes at the molecular and tissue levels (Miyata et al., 1994). I defined genes that showed sex-biased gene expression exclusively in a given brain region as "brain region-specific dimorphic genes". In this study, I found that there were 36, 38, 9 and 334 brain region-specific dimorphic genes in the POA, HY, OB and PIT, respectively. Second, I investigated when these sexually dimorphic genes emerged in the evolutionary process, because new gene functions may be required for organisms to develop new morphological traits and biochemical processes (Burki and Kaessmann, 2004; Long et al.; 2003). In this study, I show from an evolutionary perspective that sexually dimorphic genes expressed in mouse brain are also specific for particular brain regions in mice.

3.2 Materials and Methods

3.2.1 Animals and tissue sampling

I used 9-10 week-old C57BL/6J adult mice. The POA, the HY, the OB and the PIT of each of 4-5 males and 4-5 females were dissected between 1 p.m. and 5 p.m in a single sampling period. In this analysis an anterior part of the hypothalamus ahead of the optic chiasma was used as the POA and the other hypothalamic region was used as the HY. The POA, HY, OB and PIT of 4-5 males and those of 4-5 females were pooled respectively in two different tubes with RNA later (Ambion) for total RNA preparation.

3.2.2 Total RNA isolation and mRNA amplification

Total RNA was prepared from the POA, HY, OB and PIT of males and females, respectively. Total RNA was isolated using ISOGEN reagent (Nippon gene) for phase separation, and using an RNAeasy mini column (QIAGEN) for RNA purification by chromatography (Bowtell and Sambrook, 2003). The quantity and quality of the total RNA were determined by spectrophotometry using 260/280 absorption ratios. Degradation was checked by electrophoresis. Only high quality RNA, with no signs of degradation, was used in further experiments. aRNA (amplified RNA) was made by reverse transcription using Amino allyl MessageAmp™ aRNA (Ambion).

3.2.3 Fluorescent-labeling and hybridization

Ten μg of each sample of aRNA was labeled with either Cy3 mono-reactive dye or Cy5 mono-reactive dye. aRNAs were then purified using a Microbio spin column (BioRad), condensed using a Microcon YM-30 column (Millipore), and heated at 94 ℃ for fragmentation. Finally, the aRNA fragments were purified and condensed using a Microcon YM-10 column

(Millipore). I used microarrays from Hitachi Soft (30,000 probes for mouse genes). Competitive hybridization was carried out in 5x SSC, 0.5% SDS, 4x Denhardt's solution, 20% hybridization solution (Hitachi Soft), 100 μg/ml salmon sperm DNA and 10% formamide in a humidity chamber at 50 ℃ for 20 hours. The array slide was treated with consecutive washes as follows: 2x SSC-0.1% SDS at room temperature for 5 minutes; 2x SSC at 30 ℃ for 5 minutes; and 1x SSC at 30 ℃ for 5 minutes.

3.2.4 Scanning of the arrays and data analysis

Hybridization signals were scanned using a FLA-8000 scanner (FUJIFILM). Data were extracted using Array Gauge Ver2.0 (FUJIFILM) to give the mean values of the foreground intensities of Cy5 (*Rf*) and Cy3 (*Gf*) and the background intensities of Cy5 (*Rb*) and Cy3 (*Gb*) in each of probes. To select candidate dimorphically expressed genes, I calculated t-values adjusted for microarray analyses using the limma package (http://bioinf.wehi.edu.au/limma/) in the R computing environment (http://www.r-poject.org). The background-corrected intensities of Cy5 (*R*) and Cy3 (*G*) were calculated using the following formula: $R = Rf - Rb$ and $G = Gf - Gb$. The log-differential ratio ($M = log_2 R/G$) was calculated for each spot to compare the background-corrected intensity between Cy5 and Cy3. The average log ratio ($A\ value = log_2 RG$) was also calculated for each spot to investigate the intensity of a given spot. Normalization of the log-differential ratio, *M,* was carried out within each array using local weighted regression methods (LOESS) (Quackenbush, 2002), which is an intensity-dependent normalization using the fitted LOESS curve. The differences in gene expression between the sexes were evaluated using the moderated t-values (Smyth, 2004) of a given gene for four replicates in each brain region, in which the standard errors had been moderated across genes by empirical base methods; that is, shrunk towards a common value. I

chose a combination of 1.5-fold differences in mRNA expression level with a moderated t-value exceeding 99% confidence (P<0.01) as the criteria for selecting candidates of sexually dimorphic expressed genes in the mouse brain, because this combination has been shown to allow us to identify genes with biological confidence (Reinke et al, 2000). Furthermore, I cut out the dimorphically expressed genes with low signal in the microarray experiments, since the variances of the M values of the genes got larger as the hybridization signal intensities decreased, so that one might misidentify genes as being differentially expressed (Quackenbush, 2000). Genes whose A-values are less than 1 were cut out because the M values of these genes fluctuated (Supplementary Fig. 3.1). To confirm whether the candidate dimorphic genes in the brain were actually expressed in the brain I used BodyMap-Xs (Ogasawara et al., 2007; http://bodymap.jp/) and the Allen Brain Atlas (Lein et al., 2006; http://www.brain-map.org). BodyMap-Xs is a gene expression database based on the EST frequency of a given gene. First, I investigated EST counts of the candidate dimorphically expressed genes in the POA, HY, OB and PIT according to the anatomical categories of the "brain" and the "pituitary" defined by BodyMap-Xs. Next, I examined whether the candidate dimorphically expressed genes in the POA and HY were also expressed in the hypothalamus categories and whether those in the OB were expressed in olfactory bulb category, using the "expression density" of *in situ* hybridization data defined by the Allen Brain Atlas (Lein et al., 2006). The "expression density" means the number of cells in a brain region in which a given gene is expressed (Lein et al., 2006). I dropped genes that met both of the following criteria: (1) the EST count of a given gene was 0 in both anatomical categories of the brain and the pituitary according to BodyMap-Xs; (2) the "expression density" in a brain region of a given gene is less than 0.001 in the data of the Allen Brain Atlas. In the case of the POA and HY, I used the relative densities of genes expressed in the hypothalamus in the AllenBrain Atlas, because the POA is included in the HY in this

database. In the case of the OB, I used the relative densities of genes expressed in the OB. In the case of the PIT, I only used Body-Map, because the Allen Brain Atlas has no information about the PIT.

3.2.5 Quantitative Real-Time PCR

Three or four total RNA samples (including one or two samples not used in the two microarray experiments) from each brain region of female and male mice were reverse transcribed to cDNA using an ExScript reagent Kit (TaKaRa). These cDNAs were subsequently mixed with iQ SYBR Green Supermix (BioRad), a specific primer set for a given gene, and 10 ng of template, and then amplified by PCR. A comparison of gene expression between males and females in each of brain regions was performed using relative critical threshold (Ct) comparison. Ct values were calculated for each reaction and normalized to β-actin (ΔCt = X - β-actin, where X = the Ct for a given sex and gene target). The normalized Cts were then compared between sexes (ΔΔCt = M-F). Fold change (M/F) was calculated from the ΔΔCt (M/F = 2^ΔΔCt). Finally, an average of the fold change and the p-value of a t-test were calculated for 3 reactions.

3.2.6 Estimating the evolutionary emergence time of sex-related genes in the mouse brain

To understand the evolutionary process underlying the appearance of sexually dimorphic genes in the mouse brain, I investigated the proportion of sexually dimorphic genes in the mouse brain that emerged during each "period" of time for a given phylogenetic tree. I defined each period as an evolutionary time separated by epoch-making branching nodes in eukaryotic evolution, as shown by Noda et al. (2006). A total of 8 periods of eukaryotic evolution were designated (A) to (H) as shown in Figs 3.2AB.

To estimate the evolutionary emergence times of the sexually dimorphic genes in the mouse brain, I used PhyloPat database v41 (http://www.cmbi.ru.nl/pw/phylopat/41/) (Hulsen et al., 2006), which reveals the presence or absence of protein-coding genes in 26 species of eukaryotes (phylogenetic pattern) using the orthologous relationships defined by the phylogenetic trees of these genes. The phylogenetic patterns of sexually dimorphic genes in the mouse brain and 24,438 mouse genes as controls were obtained from the PhyloPat database. For each of these phylogenetic patterns, the most primitive species that possessed orthologues of these genes were identified. Next, I estimated the number of genes emerging during each of the 8 evolutionary periods in which the most primitive species existed.

3.2.7 Classification of sexually dimorphic genes in the mouse brain into molecular function categories

The molecular functions of the sexually dimorphic genes in the mouse brain were assigned according to the Gene Ontology (GO) classification. The classification of the sexually dimorphic genes in the mouse brain into molecular function categories of GO was conducted using the web tool Fatigo (http://fatigo.bioinfo.cipf.es/) (Al-Shahrour, 2004).

3.3 Results and Discussion

3.3.1 Sexually dimorphic gene expression in four brain regions

To identify candidate sex-biased genes expressed in each brain region of the mouse, I compared between males and females the amount of mRNA for 18,538 mouse genes in the POA, HY, OB and PIT by microarray analyses. I operationally defined these sex-biased genes as "sexually dimorphic genes" in the mouse brain. The dimorphic genes in the mouse brain that are expressed more highly in females were termed "female-biased genes" in the mouse brain, and those expressed more highly in males were termed "male-biased genes" in the mouse brain. Table 3.1 summarizes "dimorphic genes in the mouse brain", "female-biased genes" and "male-biased genes" in each of the four brain regions examined. Based on our criteria, 41, 44, 11 and 339 genes were selected as dimorphic genes expressed in the POA, HY, OB and PIT, respectively. These results indicate that sex differences in gene expression may exist in each of these four brain regions in the mouse.

It is expected that anatomical, biochemical and hormonal sex differences underling sexual differentiation result in sex-biased gene expression. However, despite significant phenotypic differences in the brain between the sexes in rodents (Nirao et al., 2004), it has not been revealed that there is any sexual dimorphism in the brain at the gene expression level (Rinn et al., 2004). For example, Rinn et al. (2005) showed that gene expression differences in the mouse brain are primarily limited to 6 genes that were encoded on the sex chromosome. Recently, Yang et al. (2006) demonstrated statistically that there is sex-biased gene expression in the mouse brain using a large data set of microarray experiments in the mouse brain. I showed that sex differences in gene expression may exist in each of four brain regions in mice. Thus, our data would support the idea of Yang et al. that sex differences in gene expression exist in the mouse brain.

In contrast to other organs, very few differences in gene expression between males and females were observed in adult brains (Rinn et al. 2004; Yang et al. 2006). Since the brain is a highly heterogeneous organ that shows striking physiological differences between sexes in specific regions (Arnold 2004), sex differences in gene expression within an individual region of the brain may well be masked when only the whole brain is studied (Rinn et al., 2004). Because of this concern and the limited sensitivity of microarray experiments for detecting low levels of gene expression it is likely that these authors underestimated the number of sexually dimorphic genes in the brain. In this study, for the purpose of identifying sexually dimorphic genes in mouse brains more efficiently, I focused on particular brain regions of the mouse that have been known to exhibit sex-related features in rodents. This strategy enabled us to identify more candidate sexually dimorphic genes in the mouse brain. In particular, this is the first report to show sexual dimorphism in the POA (not including the HY) and in the OB of the mouse brain.

3.3.2 Brain region specificity of sexually dimorphic gene expression

I compared the "sexually dimorphic genes" over these four brain regions in the mouse brain to examine whether there were any genes showing sex-biased gene expression specifically in only one brain region in the mouse brain. I operationally defined these genes as "brain region-specific dimorphic genes" in the mouse brain. The proportions of the brain region-specific dimorphic genes to all of sexually dimorphic genes in each of the four brain regions examined, in the mouse, are 87.8% in the POA, 86.4% in the HY, 81.8% in the OB, and 98.5% in the PIT (Table 3.2). The complete lists of all brain region-specific sexually dimorphic genes in four brain regions are available in Supplemental Tables 3.1-3.4. These results suggest that "sexually dimorphic genes" in the mouse brain show brain region specificity. This means

that the diversification of sexually dimorphic genes would be accompanied by the regionalization of vertebrate brains, and this diversification might contribute to the establishment of brain region specificity of sex-related features in vertebrate brains.

Yang et al. (2006) have shown that sexually dimorphic genes are highly organ-specific by comparing gene expression patterns between the sexes in liver, adipose tissue, muscle and brain of mice. Consequently, they suggested that the pathways involving sexually dimorphic genes would be different among these organs in the mouse. In this study, I showed that there was brain region specificity of sexual dimorphism in gene expression patterns in mice. For example, although the POA and HY of vertebrates, both of which are located side by side in the diencephalon, are considered to have related functions in the vertebrate brain (Butler and Hodos, 1996), there were only a few overlaps between the sexually dimorphic genes of the POA and the HY. Thus the pathways involving in sex-related features may be different among brain regions in mice.

Among these four brain regions in the mouse, a total of 424 genes displayed sexually dimorphic expression, but only seven genes were commonly expressed dimorphically in more than two brain regions (Supplementary Table 3.5). Three out of these seven genes, eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked (Eif2s3y), eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked (Eif2s3x), and DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked (Dby), were located on sex chromosomes (Supplementary Tables 3.1-3.4). On the other hand, most brain region-specific dimorphic genes were located on autosomal chromosomes (Supplementary Tables 3.1-3.4). This means that brain region-specific dimorphic gene expression in the brain must be due to sexually dimorphic gene regulation, because there are no differences in autosomal chromosomes between the sexes.

3.3.3 Molecular origin of sex-related genes in four brain regions in mice

It is speculated that the differences in sexually dimorphic genes among the four brain regions in the mouse that I examined were generated by differences in the evolutionary processes leading to the generation of these dimorphic genes among these brain regions. To confirm this possibility, I investigated whether there were differences in the evolutionary emergence times of sexually dimorphic genes among these four brain regions in the mouse. As already mentioned, I defined 8 evolutionary periods (Figs. 3.2AB). First, I found that the proportion of POA-specific dimorphic genes that emerged at the same period as the evolutionary appearance of sexual dimorphism in the mouse brain (period (E)) to all of the POA-specific dimorphic genes (39.4%) is significantly higher than that of all mouse genes (18.0%) (Fishers exact-test $p < 0.001$) (Fig. 3.1). In addition, the proportion of PIT-specific dimorphic genes that emerged during period (E) to all PIT-specific dimorphic genes (29.2%) is also significantly higher than that of all of mouse genes (18.0%) (Fishers exact-test $p < 0.001$) (Fig. 3.2). On the other hand, the proportions of HY-specific and OB-specific dimorphic genes that emerged during period (E) to all of the genes dimorphically expressed in the HY and the OB, respectively, are not different from those of all of mouse genes (Fig. 3.2). This means that the emergence of the orthologues of both of the POA- and PIT-specific dimorphic genes at almost the same period as the appearance of sex-related features in the brain might have contributed to the evolutionary development of sex-related features in these two brain regions.

Moreover, I classified the 13 POA-specific sexually dimorphic genes of the mouse that emerged during period (E) (Supplementary Table 3.1) according to the functional categories of GO, to clarify the characteristics of these sexually dimorphic genes that would develop sex-related features in the POA in early vertebrates. The reason that I focused on the POA in the mouse brain is that it is a critical brain region for sex-related functions, such as sexual

behavior and sexual reproduction, in vertebrate brains (Butler and Hodos, 1996; Pilgrim and Reisert, 1992). As a result, I found that 6 out of 13 (46.2%) POA-specific sexually dimorphic genes in the POA that emerged during period (E), including vasoactive intestinal hormone (Vip), islet amyloid polypeptide (Iapp), nuclear factor of activated T-cells 5 (Naft5), ectodysplasin-A receptor (Edar), Hermansky-Pudlak syndrome 6 (Hps6) and tripartite motif protein 13 (Trim13), were classified as belonging to the "protein binding" category in GO (Table 3.3). In particular, Iapp and Vip also belonged to the "receptor binding" function category, which is known to be responsible for sex-related features in the vertebrate brain (Campbell et al., 2004). Thus, it was expected that sex-related features that were involved in the ligand-receptor pathways related to these two peptide hormones were evolutionarily generated in early vertebrates. Therefore, I propose that the emergence of POA-specific sexually dimorphic genes with protein binding features in early vertebrates might specify sex-related features in the POA of the mouse brain in the evolutionary process.

Interestingly, Vip was expressed specifically in the suprachiasmatic nuclei (SCN) in the mouse brain according to the Allen brain atlas (http://www.brain-map.org/welcome.do), which is the center of circadian rhythm and is related to the transmission of circadian information from the SCN to gonadotropin releasing hormone (GnRH)-positive neurons. Vip also regulates the timing of the luteinizing hormone (LH) surge in mice (Krajnak, 2006). In humans, it has been shown that chromosomal position 7q36, which includes VIP receptor type 2 genes, has a linkage to homosexuality (Mustanski et al., 2005). VIPR2 is essential for the development of the hypothalamic suprachiasmatic nucleus in mice (Harmar et al., 2002). Therefore, Vip, which is a ligand of VIPR2, is an interesting candidate gene for sexual orientation (Swaab and Hofman, 1990). Moreover, in birds, the effect of Vip on courtship song and aggression in the male zebra finches has previously been shown (Goodson et al., 1999).

Based on these findings, the emergence of Vip during evolution might have played an important role in the formation of sex-related function in the vertebrate brain. Therefore, I validated the microarrray result for Vip in the POA by real-time RT-PCR. Vip mRNA was found to be expressed 1.64 fold higher in females than in males, in the POA of the mouse (data not shown). Thus, Vip is one candidate gene for the development of sex-related function in vertebrate brains.

3.3.4 The comparison of an evolutionary process between the POA and the HY

The POA and the HY are considered to have related functions in the vertebrate brain, since they are located side by side in the diencephalon and are connected by neuronal pathways in vertebrate brains (Butler and Hodos, 1996). However, I have shown that there are few genes that are expressed dimorphically both in the POA and the HY in the mouse in this analysis. To investigate whether these differences in sexually dimorphic genes between these two brain regions of the mouse are due to differences in the evolutionary processes involving these dimorphic genes between the POA and the HY, I compared the number of the sexually dimorphic genes that emerged in each evolutionary period between the POA and the HY (Figs 3.1, 2A and B). I found that there were statistically significant differences in the evolutionary emergence time of sexually dimorphic genes between the POA and the HY of the mouse (Fisher's exact test; $p < 0.05$).

First, as I described, I found that the POA-specific dimorphic genes that emerged during period (E) were more abundant than the HY-specific dimorphic genes that emerged during period (E) (Fig. 3.1). Second, I found that the proportion of HY-specific dimorphic genes that emerged during period (B), when the common ancestors of nematodes and mammals diverged, to all HY specific-dimorphic genes (29.4%) was higher than that of all mouse genes (17.8%), whereas the proportion of POA-specific dimorphic genes that emerged during period

(B) to all of the POA-specific dimorphic genes (12.1%) was lower than that of all of mouse genes (17.8%) (Fig 3.2B). Furthermore, HY-specific dimorphic genes that emerged during period (B) were the most of all the evolutionary period (Figs. 3.1 and 3.2B). These results suggest that the functionalization of the genes that emerged during period (B) would have been required for the evolution of sex-related features in the HY, whereas the addition of the sex-related genes in the brain that emerged at almost same time as the appearance of phenotypic sex-related features was essential for the evolution of sex-related features in the POA.

To clarify the characteristics of these sexually dimorphic genes in the HY that emerged during period (B), which would be functionalized to contribute sex-related features in the HY in vertebrates, I classified the 10 HY-specific dimorphic genes in the mouse that emerged during period (B) according to the functional categories of GO. I found that 6 out of 10 (60.0%) HY-specific sexually dimorphic genes that emerged during period (B), potassium voltage-gated channel, Shal-related family, member 3 (Kcnd3), sine oculis-related homeobox 6 homolog (Six6), BTB/POZ domain containing protein 3 (Btbd3), four and a half LIM domains 5 (Fhl5), unknown gene 2310047C04Rik and aryl hydrocarbon receptor nuclear translocator (Arnt), were classified as belonging to the "protein binding" category in GO (Table 3.4). This result means that not only the emergence of the orthologues of sexually dimorphic genes, which are categorized into the "protein binding" category in vertebrates, but also the functionalization of the orthologues of sexually dimorphic genes that are categorized as "protein binding" in vertebrates, might be required for the establishment of sex-related features in the HY of vertebrates.

It has been known that Arnt is a coactivator of the estrogen receptor (Otake et al., 2003; Brunnberg et al., 2003) and that Kcnd3 is downregulated by estrogen (Song et al., 2001). In addition, 5-hydroxyubdikeacetic acid (5-HIAA), which is a by-product of serotonin synthesis

and a candidate substrate of 2310047C04Rik (KEGG, tryptophan metabolism), has sexual dimorphism by estrogen (Rubinow et al., 1998). Therefore, the functionalization of the orthologues of these dimorphic genes in the HY has occurred by acquiring the interaction with estrogen in the evolutionary process in vertebrates, because the estrogen or androgen pathways would have evolved in early vertebrates (Baker, 2003; Dehal et al., 2002).

In summary, I show brain region specificity of the sexual dimorphism of gene expression in the mouse brain. In addition, I suggest that differences in the evolutionary processes involving sexually dimorphic genes among brain regions might have led to the development of brain region-specific sex-related features in the vertebrate brain.

Table 3.1. Distribution of genes that are differentially expressed between males and females in preoptic area, hypothalamus, olfactory bulb and pituitary gland

Brain region	Dimorphic genes	Male-biased	Female-biased
Preoptic area	41 (0.22%)	27 (0.15%)	14 (0.076%)
Hypothalamus	44 (0.24%)	18 (0.097%)	26 (0.14%)
Olfactory bulb	11 (0.059%)	10 (0.054%)	1 (0.0054%)
Pituitary gland	339 (1.8%)	108 (0.58%)	231 (1.2%)

Table 3.2. Brain region specificity of sexually dimorphic genes in preoptic area, hypothalamus, olfactory bulb, and pituitary gland

Brain region	Dimorphic genes	Brain region -specific dimorphic genes	Percentage of brain region -specific dimorphic genes (%)
Preoptic area	41	36	87.8
Hypothalamus	44	38	86.4
Olfactory bulb	11	9	81.8
Pituitary	339	334	98.5

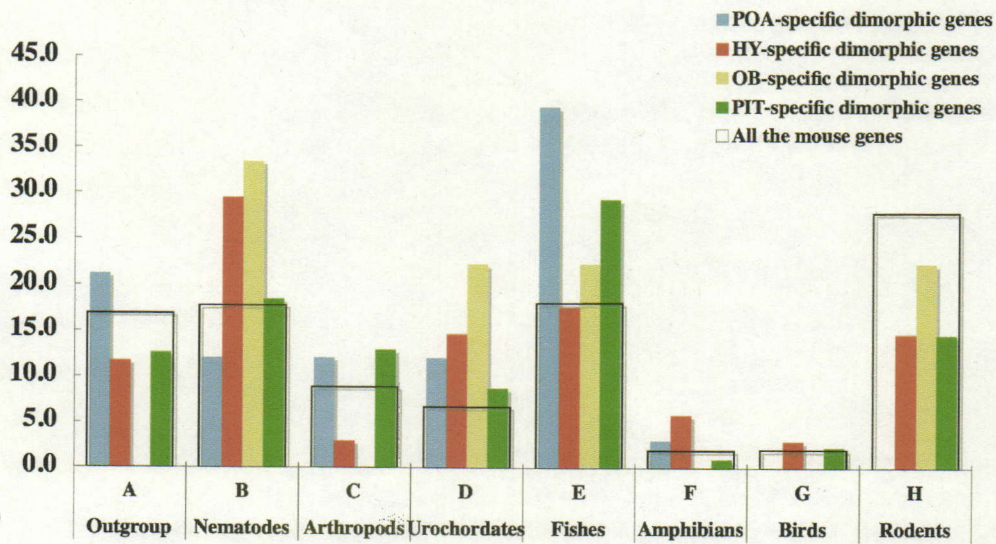

Fig. 3.1 Proportions of sexually dimorphic genes in the POA, the HY, the OB and the PIT in the mouse brain that emerged each evolutionary period of time.

Table 3.3. Molecular function categories of the POA-specific dimorphic genes that emerged during period (E)

Molecular function	Gene Symbol	Number	Percentage
protein binding	Vip Nfat5 Edar Hps6 Iapp Trim13	6	46.2
receptor activity	Edar AJ543404	2	15.4
nucleic acid binding	Nfat5 Hoxd8	2	15.4
enzyme inhibitor activity	Itih4	1	7.7
ion binding	Trim13	1	7.7
transferase activity	Parp8	1	7.7
nucleotide binding	Rasl12	1	7.7

Table 3.4 Molecular function categories of the HY-specific dimorphic genes that emerged during period (B)

Molecular Function	Gene Sumbol	Number	Percentage
protein binding	Kcnd3 Six6 Btbd3 Fhl5 2310047C04Rik Arnt	6	60.0
ion binding	Kcnd3 Fhl5 2310047C04Rik	3	30.0
nucleic acid binding	Six6 2310047C04Rik Arnt	3	30.0
transcriptional activator activity	Fhl5 Arn	2	20.0
ion transporter activity	Kcnd3	1	10.0
transferase activity	Arnt	1	10.0
GTPase regulator activity	D10Bwg1379e	1	10.0
channel or pore class transporter activity	Kcnd3	1	10.0
ligase activity	2310047C04Rik	1	10.0
oxidoreductase activity	Cryl1	1	10.0
receptor activity	Arnt	1	10.0

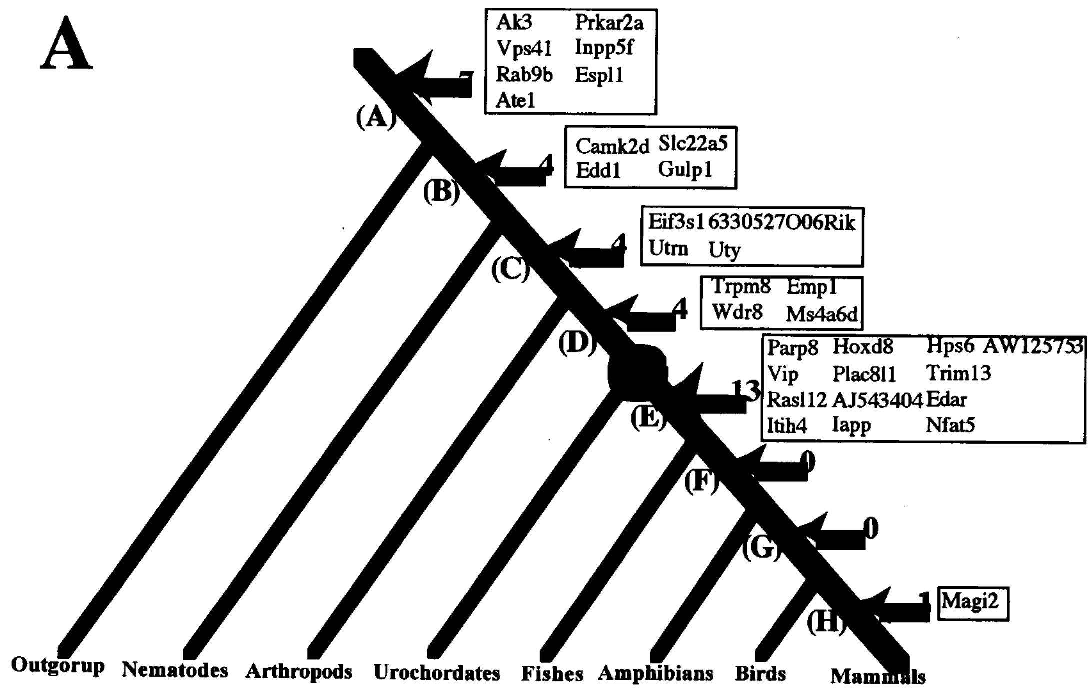

Fig. 3.2A

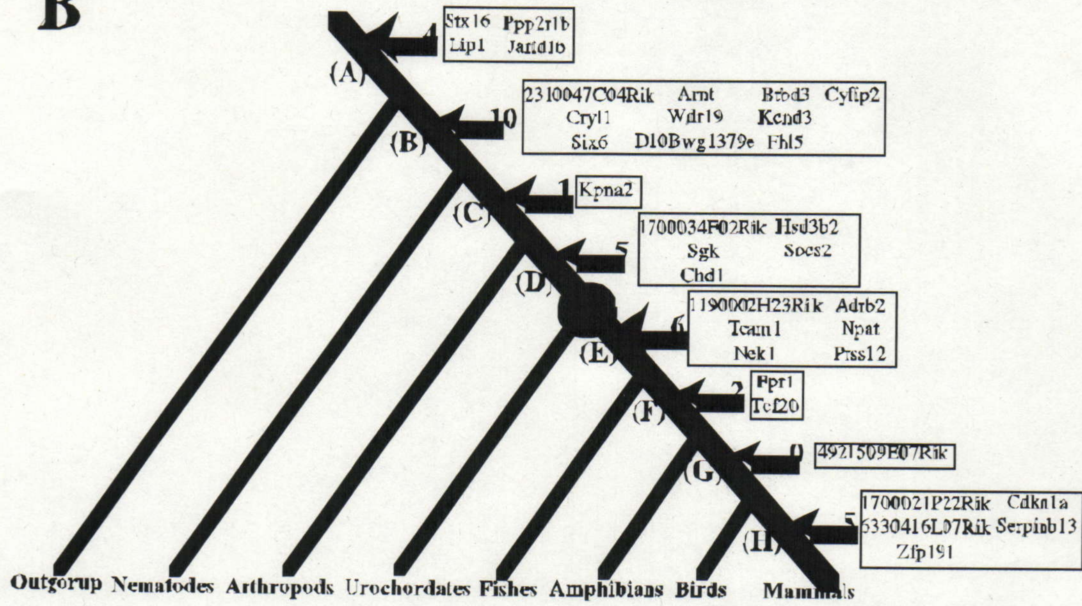

Fig. 3.2B

Chapter 4: Conclusion

In this thesis, I focused on two theames to study of the evolution of sex-related feature in the vertebrate brains.

First, I focused on the evolutionary process of sexual dimorphism in a whole brain of vertebrates at the molecular level, and I conducted genomic comparisons of a set of genes expressed in a sexually different manner in the mouse brain with all genes from other species of eukaryotes. My result showed that the orthologues of the sex-related genes in the mouse brain that emerged just before the divergence of bony fish might have essential roles in the evolution of the sexual dimorphism in the brain forming protein-protein interactions. Moreover, I found that these genes were expected to evolutionarily developed cell-cell communications that were related to sex-related feature in the vertebrate brains.

Second, I focused on each of brain region of vertebrate brains to investigate whether there are differences of sex-related features among brain regions from the evolutionary point of view, because distinct brain regions are known to have evolutionarily developed their specific functions. In fact, I showed that there exists brain region specificity of sexually dimorphism of gene expression in the mouse brain. I also found that the evolutionary emergence time of orthologues of these sexual dimorphic genes in the mouse brain have varieties among brain regions of the mouse. This result indicate that that the differences of the evolutionary process of sexually dimorphic genes among brain regions might evolutionarily developed these brain region-specificities of sex-related features in the vertebrate brain.

Finally, I could emphasize that the approach to utilize a large set of gene expression data and gene gain and loss information is useful to correlate the evolution of a given phenotypic

feature with a process of molecular evolution.

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