Morphological Analyses of Sex Differences and Age-Related Changes in C3H Mouse Midbrain

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ABSTRACT. While it is commonly hypothesized that sexual differentiation in the mammalian brain is initiated mainly by gonadal sex steroids, recent evidence has suggested that dopaminergic (DA) neurons within the rodent midbrain have sex differences independent of gonadal secretions. More recently, it has been reported that *Sry* (the sex-determining region of the Y chromosome) is directly involved in this difference. The possibility of sexual dimorphism in the mouse midbrain needs to be elucidated. In the present study, the midbrain of C3H mice, which is little understood, was investigated histologically and immuno-histoplanimetrically to reveal sexual and developmental differences. The female ventral tegmental area appeared to contain higher immunoreactivity to tyrosine hydroxylase (TH) than that of males at 11 weeks of age, whereas general histological differences between the sexes were not clearly found. The TH-immunoreactive (TH-ir) neurons within the A8, A9, and A10 mesencephalic areas were examined separately. There was the sex difference in the time period when TH-ir cell numbers significantly increased, indicating that the growth rate of midbrain DA nuclei also differs and that the midbrain DA system may trace different processes of sexual maturation between the sexes. These differences between female and male may reflect the direct regulation by *Sry* or the multiple effects of both *Sry* and sex steroids. Further experiments are needed to determine which factor forms this difference in the growth pattern in the numbers of TH-ir neurons.

KEY WORDS: age-related change, dopaminergic (DA) neuron, midbrain, sex difference, tyrosine hydroxylase (TH).

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Sex differentiation of the mammalian brain is influenced mainly by perinatal testicular steroid hormones [25]. In males, androgen (or its metabolite estrogen) initiates a program of male-specific development in many brain regions. In females, on the other hand, neural development traces female-specific processes caused by a low level of gonadal steroid hormone. These mechanisms result in morphological sexual dimorphism, which controls sexually dimorphic behaviors and functions [16]. Sex steroid hormones are associated with the constitution of several sexual dimorphisms via estrogen receptor (ER) [22]. Moreover, the sexual differentiation of dopaminergic (DA) neurons within the preoptic region of the diencephalon depends on ER [32].

However, other sex differentiation processes have been found to depend on the sex chromosomal gene directly rather than on gonadal steroid hormones in the midbrain. The cell cultures dissociated from female rat mesencephalon on embryonic day 14 (ED 14; before exposure to the gonadal steroid hormone shower) contain higher numbers of DA neurons than male cultures, and hormonal treatment does not erase this differentiation [4]. The sex-determining region of the Y chromosome (*Sry*) is transcribed in the hypothalamus, mesencephalon, and testis in adult (3-month-old) male but not in adult female mice [23]. *Sry* transcripts were also found in the male

diencephalon, midbrain, and cortex in the postnatal day (d) period between 14 and 90 [26]. These reports indicate that *Sry* has a crucial role in regulating the sex differentiation of the mammalian central nervous system (CNS). The sex difference in the mesencephalon is also found in Parkinson's disease (PD), a progressive neurodegenerative disease characterized by the selective loss of DA neurons from the substantia nigra (SN) and the concomitant appearance of motor disturbances including tremors, rigidity, and slowness of movement (akinesia). Many studies have indicated that the prevalence of PD is 1.36–3.7 times higher in men than in women [2, 5, 6, 14]. Dewing *et al.* (2003) detected at least seven murine genes that show differential expression between female and male mouse developing brains at ED 10.5 before any gonadal hormone influence [13]. For these reasons, the midbrain likely has sex differences of some kind. In fact, sexual dimorphism was found in the tyrosine hydroxylase (TH) activity of SN and in the exploratory behavior of both BALB/cJ and CBA/J inbred mouse strains [37].

Despite these many demonstrations of sex differences in the midbrain, research also indicates that there is no sex difference within mesencephalic DA nuclei. Lieb *et al.* (1996) investigated the numbers of DA neurons in the mesencephalon of male and female CBA/J mice between ED 13 and 90d, but they could not find statistically significant sex differences in the numbers of DA neurons at any time point [24]. In addition, there is a genetic control of the neuron number (including midbrain DA neurons) within inbred strains of mice [1, 39]. Presently, however, we still

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lack detailed information about sex and/or genetic strain differences within the midbrain.

There are other reasons why mesencephalic DA neurons are of interest: midbrain DA nuclei, which play crucial roles in cognition, emotion, and behavior, are also significant in clinical medicine because their functional disorder causes various neurodegenerative diseases, such as schizophrenia [27], PD [18], substance abuse [20], and disturbances in the control of motor activity and learning [3], attention [38], and other behaviors including attention-deficit hyperactivity disorder [15].

Mesencephalic DA nuclei are distributed in the A8, A9, and A10 areas [11]. A8 contains the retrorubral field (RRF); A9 consists of the SN pars compacta (SNc), SN pars reticulata (SNr), and SN pars lateralis (SNl); and A10 is composed of the ventral tegmental area (VTA), parabrachial pigmented nucleus (PBP), paranigral nucleus (PN), and central linear nucleus (CLi). Neurons in the A9 area have projective fibers toward the striatum and form the nigrostriatal dopaminergic system, which promotes the extrapyramidal function. The DA neurons within A10 project to the subcortical and cortical limbic areas as well as to the prefrontal cortex, while A8 neurons provide dopaminergic input to both the striatum and the limbic areas. A DA neuronal deficit within the A10 area contributes to drug addiction and is thought to be a point of action by schizophrenia-curative drugs [30].

In the present study, male and female C3H mice, whose midbrain is poorly understood, were examined at 3d (neonatal period), 21d (pre-puberty), and 11 week (wk) of

age (post-puberty) to elucidate the sex differences and age-related changes within the midbrain of this inbred strain during sexual maturation.

MATERIALS AND METHODS

Animals: Female and male mice of the C3H/He strain were purchased from Japan SLC, Inc. (Shizuoka, Japan) for breeding stock. The animals used for the experiments were bred in a laboratory at the Department of Animal Science at Kobe University, under a 12 hr light/dark cycle at 21–24°C and 40–60% humidity. A laboratory diet (Lab MR Stock; Nosan Corp., Yokohama, Japan) and filtered water were available *ad libitum*. The experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals of Kobe University. Nineteen mice were divided into three age groups—3d (n=5), 21d (n=8), and 11wk (n=6)—in each sex.

Tissue preparation: We gave careful attention to each experimental treatment in order to maintain the equality of all brain samples. At the time of necropsy, mice were deeply anesthetized with diethyl ether and were subcutaneously injected with heparin (1 U/g body weight) to avoid blood coagulation. After 10 min, the mice were transcardially perfused with 0.1 M ice-cold phosphate buffer (PB), pH 7.4, including 3.4 mM EDTA, followed by perfusion with ice-cold 4% paraformaldehyde in PB. The brains were post-fixed with the same fixative overnight at 4°C, dehydrated through a graded series of ethanol followed by xylene, and embedded in paraffin. Coronal serial

Fig. 1. Sagittal section of the mouse brain referred from *The Mouse Brain in Stereotaxic Coordinates* [31]. The transverse lines of *a*, *b*, and *c* correspond to the rostral, intermediate, and caudal parts of the midbrain, respectively. The lines show brief coordinates of the following figures. Line *a* corresponds to coronal photomicrographs of Fig. 2, Fig. 3A–C, and Fig. 4A, D, G, J, M, P. Line *b* corresponds to coronal photomicrographs of Fig. 3D–F and Fig. 4B, E, H, K, N, Q. Line *c* corresponds to coronal photomicrographs of Fig. 3G–L and Fig. 4C, F, I, L, O, R.

sections of 10 μm in thickness were then cut, and each section was mounted on a glass slide precoated with silane (Matsunami, Osaka, Japan).

Cresyl fast violet staining: A series of sections was made by collection at 80–100 μm intervals. After deparaffinization and rehydration, the sections were stained with 0.1% cresyl fast violet solution, which visualizes Nissl's substance for 10 min. They were then placed in distilled water (DW) and a graded series of ethanol for appropriate bleaching of dye, dehydrated with ethanol, cleared by xylene, and coverslipped with Eukitt. Each section was morphologically categorized by using a guide [31] (Fig. 1).

Immunohistochemistry: The immunoreactivity of TH was applied to visualize DA neurons. Deparaffinized tissue sections were analyzed using a Histofine Mouse Stain Kit (Nichirei Bioscience, Tokyo, Japan) for monoclonal antibody in the mouse tissues following the manufacturer's instructions. After immersion in 100% methanol including 3% H_2O_2 for 30 min at room temperature (RT) to quench endogenous peroxidase activity, the sections were incubated with the primary antibody against TH (MAB-318; Chemicon International, Temecula, CA, U.S.A.) diluted 1:1,200 in phosphate-buffered saline at 4°C overnight. Immunoreactivity was then detected by incubation with 3,3'-diaminobenzidine solution (Dako, Glostrup, Denmark) containing 0.03% H_2O_2 . The sections were counterstained lightly with 0.1% cresyl fast violet solution for 1 min. Finally, they were placed in DW and a graded series of ethanol, dehydrated with absolute ethanol, cleared by xylene, and coverslipped with Eukitt. The distribution of each DA nucleus (A8, A9, and A10) within the midbrain was classified morphologically (Fig. 1). To compare the staining intensity across different sections, each semi-serial section was captured by a digital still camera (DS-L1; Nikon, Tokyo, Japan) connected to a light microscope. The background staining intensity was manually adjusted to the regions lacking TH and Nissl staining in each microphotograph. Then the immunoexpression within the perikarya of each DA nucleus (A8, A9, and A10) was evaluated (data not shown).

Histoplanimetric analysis: The coronal semi-serial sections stained immunohistochemically were observed from the caudal level of the diencephalon to the rostral level

of the pons (Fig. 1) at magnifications of $\times 200$ using an Olympus light microscope. The first sampling tissue section for counting was taken when the most rostral TH-immunoreactive (TH-ir) cells were observed in the midbrain unilaterally (right side), and all the following sections were taken at intervals of 80–100 μm from the previous one. Then only neurons containing perikarya clearly stained for TH within the right side of the A8, A9, and A10 areas were manually and separately counted through the entire midbrain, and the total (A8 + A9 + A10) number of neurons was calculated.

Statistical analysis: Unpaired *t*-test (StatView version 5.0; SAS Institute, Cary, NC, U.S.A.) was applied to determine the statistical differences in the TH-ir cell numbers by sex and age. Analyses were considered statistically significant at $P < 0.05$.

RESULTS

The age-related changes found in both cresyl fast violet and immunostained sections are shown in Figs. 2 and 3. In general, all of the neurons we investigated were multipolar, which are most typical in the CNS, and the perikarya and cellular nuclei increased slightly in size as the mice developed. The volume of nuclei also increased concomitantly with brain development. In the cresyl fast violet-stained sections, the nuclei composed of round or ovoid cells with short dendrites were arcuate hypothalamic nuclei (Arc), interpeduncular nuclei (IP), PN, CLi, and pontine nuclei (Pn). On the other hand, nuclei whose perikarya (with comparatively long dendrites) appeared stellate or pyramidal were SNc, SNl, RRF, and VTA. Extremely large cells were distributed within oculomotor nuclei (3N) and red nuclei (Rn) in both sexes. These findings were common between the sexes. The findings observed in TH-immunostained sections of both sexes were as follows: TH-ir cells contained deeply stained perikarya and round unstained nuclei. SNc seemed to consist of the largest TH-ir cells in the midbrain DA nuclei (Fig. 3). CLi and PN appeared to contain comparatively small TH-ir cells, and their immunostained fibers were comparatively short (Fig. 3D-I). The regions in which TH-ir cells projected comparatively long fibers were observed in SNc,

Fig. 2. Age-related changes of rostral midbrain in cresyl fast violet-stained sections corresponding to the transverse line *a* in Fig. 1. Coronal photomicrographs show changes at 3d (A), 21d (B), and 11wk (C). The size of perikarya and cellular nuclei constituting each neuronal nucleus increases slightly, as do the contents of neuronal nuclei in parallel with brain development. Bar=100 μm .

Fig. 3. Age-related changes of DA nuclei within rostral (A-C), middle (D-F), and caudal (G-L) levels of midbrain containing TH-ir cells at the age of 3d (A, D, G, and J), 21d (B, E, H, and K), and 11wk (C, F, I, and L). TH-ir perikarya become slight larger, and the volumes of TH-ir nuclei increase in parallel with brain development. Bar=100 μ m.

SNr, and RRF (Fig. 3). The cell bodies were pyramidal or spindled within SNc, SNr, and VTA (Fig. 3A-F) but were round and ovoid within CLi and PN (Fig. 3D-I).

The immunohistochemical comparison between the sexes is shown in Fig. 4. Although the greatest individual differences were observed at 3d, the differences did not appear to depend on sex (Fig. 4A-F). Nor was any sexual dimorphism more apparent than individual differences in 21d mice (Fig. 4G-L). In the 11wk mouse midbrain, female VTA appeared to contain higher immunoreactivity of TH than male VTA (Fig. 4M-R). This sex difference seemed to contribute to the significant increases in A10 TH-ir cells at

3d, 21d, and 11wk.

The numbers of TH-ir neurons within midbrain DA nuclei (A8, A9, A10, and total) and the statistical outline of this study are summarized in Figs. 5 and 6, respectively. There were no significant sex differences in the number of TH-ir neurons when they were tested between age-matched groups. However, when the increment in TH-ir cell numbers was examined between 3 and 21d and between 21d and 11wk, there was the sex difference in the time period when the numbers significantly increased (Figs. 5 and 6); the numbers of female A9, A10, and total TH-ir neurons increased significantly between 3 and 21d (Figs. 5B, C, D,

Fig. 4. Immunohistochemical comparison between female (A-C, G-I, and M-O) and male (D-F, J-L, and P-R) mice at 3d (A-F), 21d (G-L), and 11wk (M-R). Coronal figures show rostral (A, D, G, J, M, and P), middle (B, E, H, K, N, and Q), and caudal (C, F, I, L, O, and R) levels of midbrain. Although individual differences are observed within the midbrain of 3d mice, sex differences appear to be independent of the sex. Bar=200 μ m.

Fig. 5. TH-ir neurons counted within semi-serial sections of A8 (A), A9 (B), A10 (C), and total (D) in female (solid column) and male (open column) 3d, 21d, and 11wk mice. Significant differences are not detected in the number of TH-ir neurons between the sexes when we tested between age-matched groups, while the time period in which the number significantly increased differs between females and males. In a comparison between 3d and 11wk mice, all nuclei except for male A8 area significantly increase TH-ir neuron numbers. The A9 and A10 areas of both sexes and the female A8 area show a significant increase of $P < 0.01$. Values are means $\pm$ SD. *: $P < 0.05$, **: $P < 0.01$.

	3d→21d	21d→11wk
A8	Significant difference was not detected.	↑ ($P < 0.05$)
A9	↑↑ ($P < 0.01$)	↑ ($P < 0.05$)
A10	↑ ($P < 0.05$)	↑ ($P < 0.05$)
Total	↑↑ ($P < 0.01$)	Significant difference was not detected.

↑ Male
 ↑ Female

Fig. 6. The statistical outline of this study. The significant increase of the numbers of TH-ir neurons is detected and its increment patterns differ between sexes. It is notable that females show significant increases within all nuclei while males do so only within the A9 area.

and 6); and the numbers of female A8, A10, and male A9 TH-ir neurons increased significantly between 21d and 11wk (Figs. 5A, B, C and 6). In the A8 area, the numbers of TH-ir neurons increased significantly from 21d to 11wk in females only, while neither sex showed significant increases in the numbers of TH-ir neurons between 3 and 21d (Figs. 5A and 6). The numbers of TH-ir neurons within the A9 area reached to adult level before 21d in female mice, whereas in males the numbers did between 21d and 11wk. Among females, the numbers of A10 TH-ir neurons

increased significantly from 3 to 21d and from 21d to 11wk, whereas among males the number did not significantly change except between 3d and 11wk (Figs. 5A-D and 6). The numbers of TH-ir neurons within the female A8 area seemed to reach to adult level after than in males.

DISCUSSION

The present study demonstrates the postnatal profiles of age-related changes within the midbrain of the female and

male C3H mouse. The rationale for using this strain in this study is, although several other reports have shown the data related to both female and male midbrain DA nuclei by using strains CBA/J [24], BALB/c [19], C57BL/6J [17], and ddY [35], there is little information about C3H mice, which are extensively used as experimental animals. The profiles in this study could provide a framework for further investigations of the C3H mouse midbrain.

The VTA of female mice at 11wk contained higher levels of TH-ir activity than that of males in the immunohistochemical analysis. While the number of TH-ir cells did not significantly differ between the sexes, there was a female-dominant tendency in TH-ir cell numbers within A10 ($P=0.073$). However, two other immunostaining groups—that at 3d and that at 21d—did not show any sex differences. The numbers of TH-ir cells within the A8, A9, and A10 areas peaked at 11wk in both female and male mice. Some studies have shown decreased TH-ir cell numbers within the midbrain through the ontogenetic period. Tepper *et al.* (1994) reported a decrease in TH-ir profile counts in the rat SNc between 1 and 14d, which they interpreted as cell loss [36]. Lieb *et al.* (1996) also demonstrated a decrease in profile density but an increase in total numbers of TH-ir cells [24]. However, in the present study, we did not detect a decrease in TH-ir cell numbers at any time point. This discrepancy may be attributable to differences in species and strains between our study and the previous ones. Alternatively, the number of TH-ir neurons may decrease at time points other than 3d, 21d, and 11wk. The numbers of DA neurons in A8, A9, and A10 did not significantly differ between females and males at any time point investigated, supporting the report of Lieb *et al.* (1996) [24]. However, there was the sex difference in the time period when the DA neuron number significantly increased. This means that the development rate of each DA nucleus within the midbrain differs between sexes. This, in turn, has two implications. First, statistically significant sex differences in the numbers of TH-ir neurons may be found at time points other than 3d, 21d, and 11wk. Second, sex differences may exist within the midbrain and may reflect the growth rate in DA neuron numbers. The brain of the 11wk mouse is mature enough to be unsusceptible to sex steroid hormones, whereas the brain at 3 and 21d may be affected by gonadal secretions in male mice, which may cause a sex difference in which the growth rate in the number of TH-ir neurons increases.

Many reports have demonstrated functional sex differences within the midbrain DA system affected by sex steroid hormones through the intermediary of AR and/or ERs. Both ER α - and ER β -ir were expressed in the SNc and VTA of young adult female mice [28]. There are double-immunoreactive neurons of AR and TH within the SN, VTA, and RRF of rat [21]; these neurons project to either the amygdala or the nucleus accumbens [10]. ER β -ir nuclei were found only in neurons, more specifically, within subsets of both DA and nondopaminergic neurons in the dorsal VTA, the PBP, the SNl, and the RRF [9]. Studies

suggesting functional sex differences within the midbrain have also been reported. Estrogen acts as a neuroprotective agent differentially between the sexes in neurodegenerative rats administered 6-hydroxydopamine (6-OHDA). Estrogen replacement therapy reduces the lesion size in females but increases it in males [29]. Analysis of DA cell loss within A9 after injury by 6-OHDA administration shows that males are more susceptible to toxicity; female rats have significantly less DA cell loss and respond to 6-OHDA with a significantly higher degree of behavioral recovery after the injury [34]. A9 lesions significantly deplete neostriatal dopamine, which is highly correlated with mount latency, mount rate, ejaculation latency, and ejaculation frequency in males [7]. A10 DA neurons, which are sensitive to gonadotropin-releasing hormone, are related to the regulation of lordosis in female rats [33]. These reports strongly suggest that the midbrain DA system includes sex differences underlying the pathogenic mechanism as well as functional sexual dimorphisms. Given that there are sexual dimorphisms in AR and/or ERs, the sensitivity of gonadal secretion will be very different between the sexes. In the present study, the sex differences observed in the maturation rate likely reflect functional sex differences within the midbrain DA system involving cognition, emotion, and behavior.

Chromosome Y genes may also affect the sex-specific development and the functions of the midbrain DA system. One of the genes presumed to cause the male-specific increment pattern of TH-ir neurons is *Sry*, which is expressed specifically in the brains of adult male mice [13, 26]. The transcript product of the gene is specifically detected in the TH-ir neurons within the SN of the adult male rodent and involves the regulation of mesencephalic TH [12]. An experiment using cell cultures dissociated from midbrains of female and male mice also indicates *Sry*'s involvement in the regulation of DA neurons [8]. The lack of a statistically significant increase in the numbers of TH-ir neurons from 3 to 21d in males may reflect individual differences in the complicated mechanisms regulated by male-specific factors, such as neonatal testicular secretion and chromosome Y genes. In females, the absence of male-specific factors might induce 'uniformly' developing patterns of TH-ir neurons that showed statistical significance.

In conclusion, we detected age-related changes within the midbrain of female and male C3H mice and showed sex-specific development patterns in the numbers of TH-ir neurons there, suggesting that the midbrain DA system may be sexually dimorphic in the maturation process. These differences may reflect the direct regulation of *Sry* or the multiple effects of both *Sry* and sex steroid hormones. We have not yet determined clearly which factor forms sexual dimorphism in the midbrain. To elucidate the mechanism underlying female and male development within midbrain DA nuclei, we need to perform further experiments applying gonadectomy and the administration of sex steroid hormones. We also have to determine the meaning of the

sex-specific increment patterns of TH-ir cells. In light of the fact that there is genetic variation in midbrain DA neuron numbers, the influence levels of hormonal and/or genetic factors may differ according to the inbred strain.

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