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Sexual Dimorphism in Monoamine Metabolism in BrdU-Treated Rats Showing Behavioral Dopamine Hypersensitivity: An Animal Model of Schizophrenia

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1. Introduction

A nucleotide analog 5-bromo-2'-deoxyuridine (BrdU) is a genotoxic compound that is incorporated into DNA [1]. When rodent fetuses are exposed to BrdU prenatally, the cortical development is profoundly affected. Cortical abnormalities induced by prenatal BrdU are shown as a reduction in thickness of the cerebral cortex [2]. An induction of apoptotic cell death in the mouse and rat fetal brain [3, 4] and disturbance to normal migration that induces abnormal composition of cortical glutamatergic or GABAergic neurons have been demonstrated [4]. Mouse data suggest that the prenatal BrdU treatment induced apoptotic phenomenon without sex difference (Figure 1).

Adult rats that were prenatally treated with BrdU show locomotor hyperactivity. The hyperactivity was observed in both male and female rats and was characterized as an increase in spontaneous motor activity during dark cycles observed in home cages [5] and novelty-induced hyperlocomotion in the open-field [5 - 8]. This abnormal behavior is exacerbated by the treatment with dopamine (DA) agonist, methylphenidate, which indicates that animals acquired hypersensitivity to dopaminergic stimuli [5, 8]. Recently proposed animal models for schizophrenia that knock-out candidate genes for this disease, such as neurotensin receptors [9] or calcium/calmodulin-dependent kinase II alpha [10], show hyperactive phenotypes and abnormal striatal DA function while mice overexpressing DA D₂ receptors in the striatum show unaltered locomotor activity [11]. In a major psychiatric disorder schizophrenia, disturbance in cortical development and subcortical dopaminergic abnormality have been proposed [12] and they induces hypothetical

pathology in this disease “a prefrontal hypodopaminergia and a subcortical hyperdopaminergia” [13]. We propose that prenatal BrdU-treated rats is an animal model of schizophrenia based on findings that 1) the malformation of the cerebral cortex and 2) DA hypersensitivity.

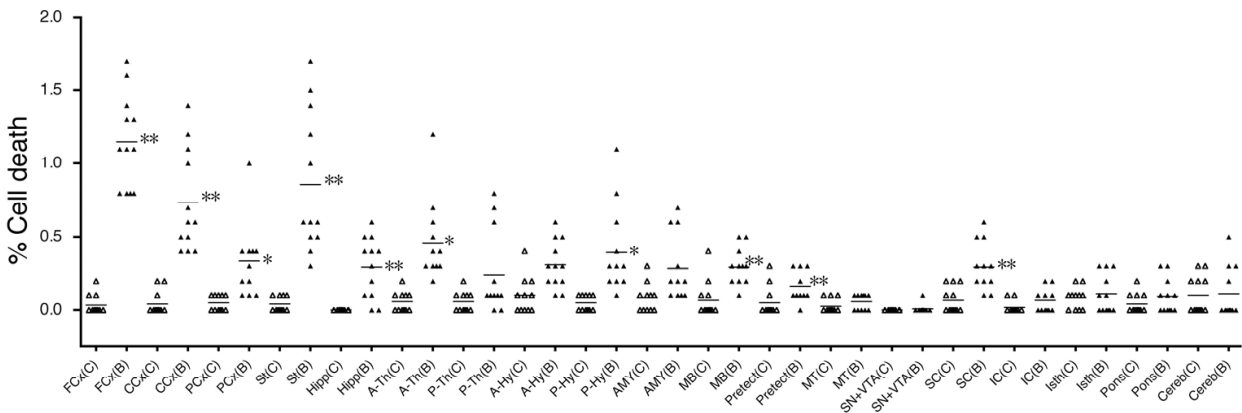


Figure 1. Incidence of cell death in fetal mouse brains on the embryonic day 12 after the treatment with BrdU at the embryonic day 11 without indenfyng sex. Apoptotic features were inspected under Nissl stain. Data were indicated as percents of numbers of apoptotic cells; C and B in parentheses indicates control and BrdU-treated animals. Horizontal bars indicate means. Significant increases in the numbers of apoptotic cell death were indicated as asterisks: *, **, P < 0.05, 0.01, respectively vs. controls. Results suggest that apoptotic cell death occurred similarly in fetal brains between males and females because values in % cell death in BrdU-treated brains in sample of mixed males and females scattered without clustering. FCx, the frontal cortex; CCx, the central cortex; PCx, the posterior cortex; St, the striatum; Hipp, the hippocmpus; A-Th, the anterior thalamus; P-Th, the posterior thalamus; A-Hy, the anterior hypothalamus; P-Hy, the posterior thalamus; AMY, the amygdala; MB, the mamillary body; Pretect, the pretectum; MT, the mecencephalic tegmentum; SN+VTA; The sunbtantia nigra + the ventral tegmental area; SC, the superior colliculus; IC; the inferior colliculus; Isth, the inthmus; Cereb, the cerebellum.

Sexual dimorphism in animal models of mental disorders is interested theme to be explored because sexual difference has been reported in clinical features in such disorders including schizophrenia. Emergence of abnormal symptoms in human mental illnesses has been known to be relevant to the beginning of puberty and this phenomenon appears to be influenced by gonadal hormones [14]. Gender differences in the age-of-onset and prevalence of mental disorders indicate an involvement of sex hormones in schizophrenia. Schizophrenia is more likely to emerge during adolescence or shortly afterwards and the first symptoms of schizophrenia emerge earlier in men than women [15]. This is more direct evidence for the involvement of sex steroids is the observation that adult women with schizophrenia have worse psychiatric symptoms and increased rate of relapse when estrogen levels are low in premenstrual period, postparturition or postmenopausal period [16]. Furthermore, estrogen has been shown to improve recovery from acute psychotic symptoms and to reduce both positive and negative symptoms of schizophrenia [17]. In adult males with schizophrenia testosterone levels are reduced and are inversely correlated with negative symptoms [18]. Altered expression of estrogen receptor alpha putatively rendering them sex-steroid unresponsive or insensitive has been reported [19]. Blunted sex

steroid signaling has implications for both males and females with schizophrenia, as testosterone can be converted directly into estrogen by brain aromatase and the lack of functional estrogen receptors is found in both males and females with schizophrenia.

The changes in hormone levels that accompany sexual maturation in puberty are critically involved in the development of the monoaminergic system [20]. In animal studies, in addition to the well-known modulatory effects of estrogen or progesterone on DA neurotransmission or DA-related behavior [21 - 25], it has been reported that neuronal systems whose dysfunction mediates the emergence of psychotic-like behavior develops after the emergence of puberty, suggesting a role for gonadal hormones in the expression of pathological phenotypes [26, 27]. A study of rhesus macaques has demonstrated that intact adult animals display attenuated prepulse inhibition than animals given prepubertal castration [28]. In addition, results from studies of sexual dimorphic effects of prenatal stress in rats imply that the developing brain of female fetuses is less sensitive to maternal stress exposure than male ones, however, enhanced aggressive behavior or disturbed estrous cycle are observed [29]. Neonatal stress manipulation, maternal deprivation from pups, increases DA and 5-HT levels in the striatum in adulthood and the magnitude of changes are greater in males than females [30].

In this chapter, we introduce the development of this animal model, their behavioral and neurochemical characters. Then, we evaluate recent obtained data of sexual dimorphic changes in DA and serotonin (5-HT) metabolisms in the prenatally BrdU-treated rats, and effects of gonadectomy that was performed during the prepubertal period.

2. Methods

2.1. Animals, drug treatments and gonadectomy

Sprague-Dawley rats were purchased from Charles River Laboratories (Tsukuba, Japan). They were housed in metal cages in a room in which the temperature and relative humidity were controlled at $24 \pm 1^\circ\text{C}$ and $50 \pm 5\%$, respectively. Lights were turned on from 0700 to 1900 h daily, and food and tap water were freely accessed. At 11 weeks of age, female rats were cohabited overnight with males. Females with sperm in their vaginal smears were regarded as pregnant, and were randomly assigned to the control or test groups. The day when the insemination was confirmed was designated as GD 0. BrdU (Sigma, St. Louis, MO) was suspended in 0.5% sodium carboxymethyl cellulose (CMC Na) and intraperitoneally administered to the test animals at 1300 h daily on GD 9 through 15. Females in the test group received a BrdU dose of 50 mg/kg, whereas control females subjected to the same regime received 0.5% CMC Na (5 ml/kg). The dosages were based on body weight on GD 9. The day of birth was designated postnatal day 0 (PND 0). On PND 1, each litter was reduced to eight animals; four males and four females. On PND 21, all offspring were weaned. At 10 weeks of age, one male and female animal obtained from an independent litter were sacrificed by decapitation and their brains were removed between 1600 and 1800 h. Each brain was subsequently dissected on ice. All tissues were stored at -80°C until the assay. We arranged other groups to investigate the effect of gonadectomy: the BrdU/GDX and BrdU/non-GDX

groups. Female pups obtained from BrdU-treated pregnant rats were used for this investigation. Female offspring assigned to the BrdU/GDX group were gonadectomized at 21 days of age, while animals assigned to the BrdU/non-GDX group received a sham operation.

2.2. Biochemical measurements

DA, 5-HT and their major metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxy-3-indolacetic acid (5-HIAA) were determined by reverse-phase high-performance liquid chromatography with electrochemical detection (HPLC-ECD) as previously described [31]. Briefly, the brain tissues were homogenized in 0.1 M perchloric acid containing 1 mM EDTA and 2 mM Na₂S₂O₈. Chloroform was added, and the mixture was then centrifuged at 11752 × g for 30 min at 4°C. The supernatant was removed and injected into the HPLC system. The HPLC system consisted of an EP-300 pump (Eicom Co., Kyoto, Japan), an ODS C18 reverse-phase column (Eicompak MA-5ODS, 4.5 × 150 mm; Eicom Co.) and an ECD-300 electrochemical detector (Eicom Co.) with a graphite working electrode maintained at +0.7 V with respect to an Ag/AgCl reference electrode. The mobile phase was 0.035 M sodium acetate–0.05 M citric acid (pH=3.9) containing 1.1 mM octanesulfonate, 8.3 mM EDTA, and 15% methanol (v/v). As indices of DA turnovers, DOPAC/DA and HVA/DA ratios were calculated. As an index of 5-HT turnover, 5-HIAA/5-HT ratio was calculated.

2.3. Evaluation of sexual behavior in offsprings

At 15 weeks of age, male animals were presented with an ovariectomized female brought into sexual receptivity by sequential treatment with estradiol benzoate and followed progesterone. Masculine sexual behavior was evaluated in the numbers of mounts and latency to the first mount. After this observation, each male was cohabited with an treated female to evaluate fertility.

2.4. Statistical analysis

Data are shown as means ± S.E.M. Student's *t*-tests and one-way ANOVA were applied for comparisons between two groups and three groups, respectively. Monoamine contents were formulated into percent values of controls before analysis for differences between Male-BrdU, Female-BrdU and Female-BrdU-GDX because each group had independent controls. Post-hoc Tukey/Kramer tests was performed accompanied with one-way ANOVAs. Two-way ANOVAs were applied to analyze two independent factors (Sex and BrdU) simultaneously. *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Behavioral and pharmacological aspects of prenatally BrdU-treated rats

Behavioral and pharmacological aspects of prenatally BrdU-treated rats are summarized in Table 1. Spontaneous locomotor activity in the open-field was elevated both in male and female BrdU-treated rats when it was measured for 3 and 60 min [5, 8]. Activity in home

cages of BrdU-treated animals was also elevated both in males and females but this hyperactivity was found in the dark cycle but not in the light cycle. The characteristic hyperactivity in the open-field induced by prenatal BrdU treatment was challenged to be influenced with dopaminergic, serotonergic and noradrenergic agents; methylphenidate (DA agonist), SCH23390 (DA D₁ receptor antagonist), sulpiride (DA D₂ receptor antagonist), NAN190 (5-HT_{1A} receptor antagonist), ketanserin (5-HT_{2A} receptor antagonist), paroxetine (selective serotonin reuptake inhibitor) and desipramine (noradrenaline reuptake inhibitor) [5, 6]. The data were obtained in male rats. Methylphenidate facilitated hyperlocomotion shown in BrdU-treated rats dose-dependently. The dose of methylphenidate (1 mg/kg) that did not stimulate locomotion in controls elevated locomotor activity of BrdU-treated rats [5, 8]. SCH23390 and NAN190 decreased activity in BrdU-treated rats but similar changes were also found in control rats. Effects of sulpiride and ketanserin on hyperactivity of the BrdU-treated rats were not certain. However, paroxetine and desipramine suppressed hyperlocomotion of BrdU-treated rats dose-dependently without influencing the activity of controls [5]. These results suggest that BrdU exposed to fetus induced similar behavioral phenotype in male and female animals. The BrdU-treated animals seem to show behavioral hypersensitivity to a DA agonist. Inhibition of reuptake of 5-HT or noradrenaline seems to suppress locomotor activity specifically in BrdU-treated rats rather than antagonists to DA or 5-HT receptors.

	Male	Female
Spontaneous locomotor activity		
Open field test (3 min)	↑	↑
Open field test (60 min)	↑	↑
Activity in home cages (light cycle)	→	→
Activity in home cages (dark cycle)	↑	↑
Effects of pharmacological challenges on BrdU-induced hyperactivity (data obtained from male rats)		
Methylphenidate (dopamine agonist)	↑	
SCH23390 (dopamine D ₁ receptor antagonist)	↓	
Sulpiride (dopamine D ₂ receptor antagonist)	→ or ↑	
NAN190 (5-HT _{1A} receptor antagonist)	↓	
Ketanserin (5-HT _{2A} receptor antagonist)	→	
Paroxetine (selective serotonin reuptake inhibitor)	↓	
Desipramine (noradrenaline reuptake inhibitor)	↓	

Table 1. Behavioral and pharmacological aspects of prenatally BrdU-treated rats

3.2. Effects of prenatal BrdU treatment on DA and 5-HT and their metabolites in male and female offsprings

In the frontal cortex, brain contents of 5-HT and 5-HIAA seem to elevate in BrdU-treated females but not in BrdU-treated males (Figure 2A). In the striatum, DA and DOPAC contents significantly decreased in BrdU-treated males while DA contents significantly elevated and DOPAC contents showed control levels in BrdU-treated females (Figure 2B).

BrdU-treated male rats shows prominent increases in striatal 5-HT and 5-HIAA while 5-HT levels were mildly elevated and 5-HIAA levels were comparable to controls' values in BrdU-treated females (Figure 2B). Significant reductions in DA and a significant reduction and a tendency to decrease in DOPAC contents were found in the hypothalamus of male and female BrdU-treated rats (Figure 2C). There were a significant reduction in HVA in females and a significant reduction in 5-HIAA in males in BrdU-treated animals in the midbrain (Figure 2D).

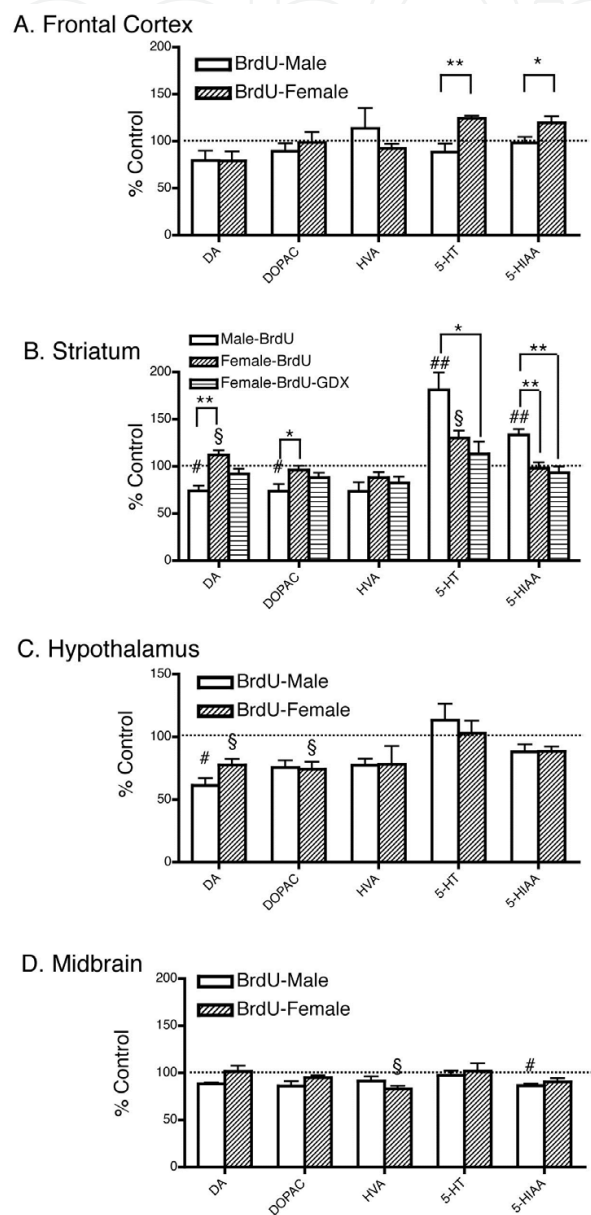


Figure 2. Changes in tissue contents of monoamines and their metabolites in prenatally BrdU-treated rats. Data are indicated as percent values of controls. Statistical significance in difference between controls and BrdU-treated animals is indicated as symbols located at the top of columns; #, $P < 0.05$ vs. male controls; ##, $P < 0.01$ vs. male controls; \$, $P < 0.05$ vs. female controls. Statistical significance in percent values between BrdU-treated males (Male-BrdU), BrdU-treated females (Female-BrdU) and BrdU-treated females that are given gonadectomy (Female-BrdU-GDX) is indicated as asterisks located between columns; *, $P < 0.05$; **, $P < 0.01$.

3.3. Effects of prenatal BrdU treatment and sex on ratios of DOPAC/DA, HVA/DA and 5-HT

There was no significant effect of BrdU or Sex on DOPAC/DA, HVA/DA or 5-HT in the frontal cortex (Figure 3A). In the striatum and the midbrain, significant effects of Sex on all measured turnover ratios and a significant effect of BrdU on 5-HIAA/5-HT ratio were detected (Figure 3B and D). There were significant effects of Sex on DOPAC/DA and 5-HIAA/5-HT and a significant effect of BrdU on 5-HIAA/5-HT in the hypothalamus (Figure 3C). Significant interaction of the two independent factors Sex and BrdU was detected in any measured values. These data suggest that prenatal BrdU affects 5-HT turnovers in the striatum, hypothalamus and midbrain in male and female offsprings in same manner. These brain regions seem to show intrinsic sexual differences in DA and 5-HT turnovers.

3.4. Sexual dimorphism in the effects of prenatal BrdU treatment on DA and 5-HT metabolism

Statistical analysis indicated significant sexual dimorphic effects of prenatal BrdU on monoamines in the frontal cortex and the striatum but not in the hypothalamus and midbrain. There were significant differences in percent changes of 5-HT and 5-HIAA compared with control levels between male and female offspring in the frontal cortex (Figure 2A). In the striatum, significant differences in percent changes of DA, DOPAC and 5-HIAA compared with control levels were detected between male and female offspring in the striatum (Figure 2B).

3.5. Effects of prepubertal gonadectomy of BrdU-treated females on striatal monoamines

Results were summarized in Table 2. No statistical difference in DA and DOPAC levels were found in the BrdU-treated females that gave gonadectomy prepubertally compared with the BrdU-treated male and female rats without gonadectomy although marked differences in BrdU-induced changes were found in DA and DOPAC levels between male and female groups (Figure 2B). Significant differences in 5-HT and 5-HIAA levels were found in the BrdU-treated females with gonadectomy compared with the BrdU-treated males (Figure 2B), which were similar changes to the BrdU-treated females without gonadectomy. These results indicate abolishment of sexual dimorphism by prepubertal gonadectomy in effects of prenatal BrdU treatment on the DA system but not in the 5-HT system in the striatum.

3.6. Disruption of sexual behavior in male offsprings from BrdU-treated dams

Males in the BrdU group showed the significantly lowered number of mounts and aberrant latency of the first amount, which resulted in a significant decrease in the copulation and fertility [7].

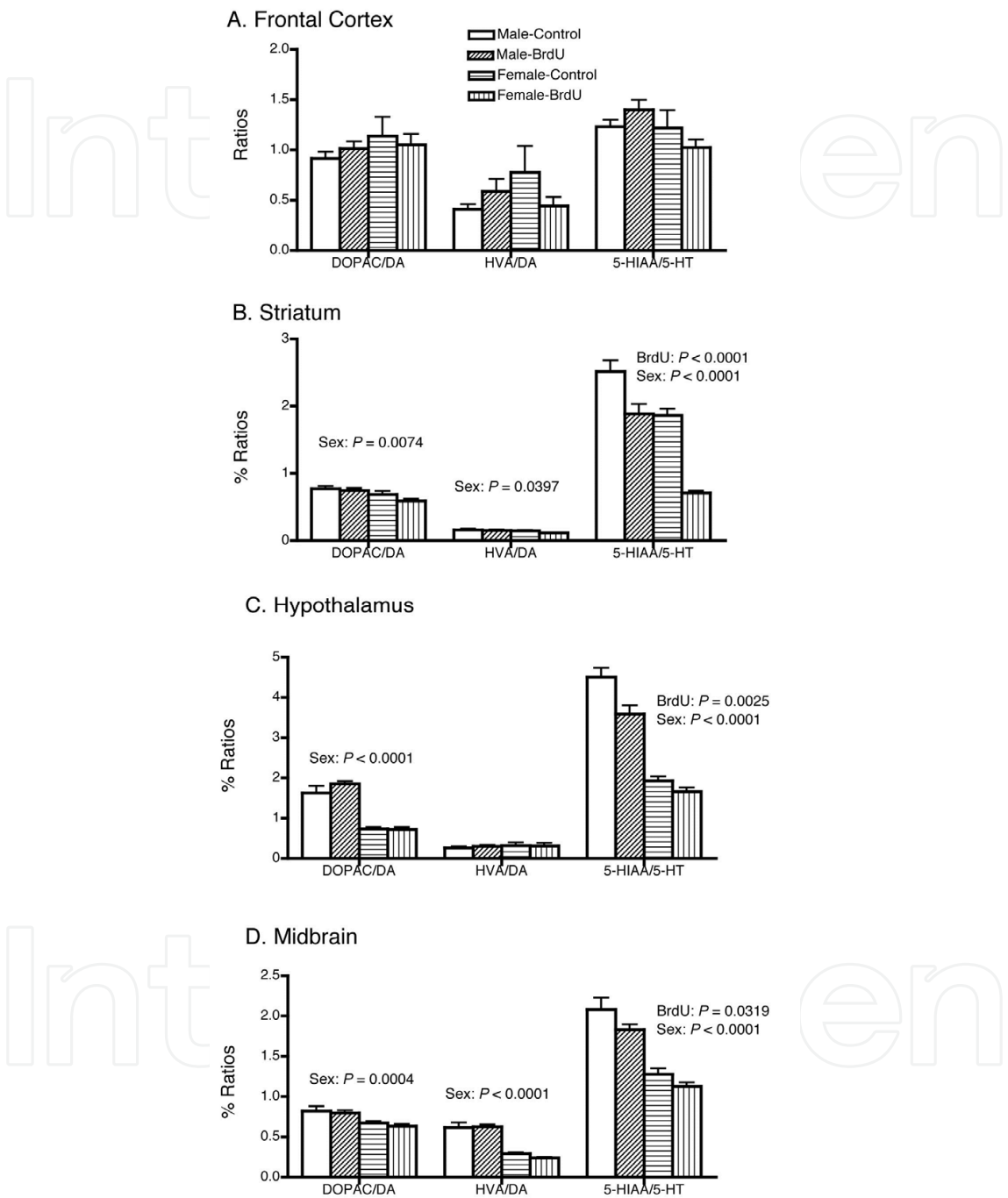


Figure 3. DOPAC/DA, HVA/DA and 5-HIAA/5-HT ratios in male controls (Male-Control), BrdU-treated males (Male-BrdU), female controls (Female-Control) and BrdU-treated females (Female-BrdU). *P* values in applied two-way ANOVAs as factors of SEX and BrdU are incorporated in figures when results were statistically significant.

Monoamines and metabolites	Male-BrdU	Female-BrdU	Female-BrdU + prepubertal gonadectomy
DA	↓↓	↑	→
DOPAC	↓↓	→	→
HVA	→	→	→
5-HT	↑↑	↑	→
5-HIAA	↑↑	→	→

Table 2. A summary of sexual dimorphic effects of prenatal BrdU on the monoamine metabolism in the striatum

4. Discussion

The treatment with BrdU in the mid-pregnancy induced apoptotic cell death in fetal brains in rodents without sex difference. Offspring from prenatal BrdU-treatment showed prominent hyperactivity in familiar or novel environment after maturation, which was observed both in male and females. However, sexual behavior was disrupted in male offspring when they were prenatally treated with BrdU. In this animal model, sexual dimorphism in monoamine metabolism was revealed. Most obvious differences in monoamine metabolism between males and females were found in DA contents in the adult striatum; a decrease in males and an increase in females. While DA levels seem to be reduced in the frontal cortex similarly in males and females with prenatal BrdU treatment. Another sexual dimorphic effect was changes in 5-HT in the striatum and the frontal cortex. Increases in 5-HT were also found in the striatum in both sex but the magnitude in the changes were larger in males rather than in females. Increased 5-HT levels in the frontal cortex were obvious in females treated with BrdU prenatally but not in males. Therefore, prenatal BrdU treatment affects the striatal DA and 5-HT system most seriously in males. Effects of prenatal BrdU on the frontal cortical DA were moderate and the magnitude was similar in males and females. Cortical 5-HT was changes only in females.

Results from the study of gonadectomy during prepubertal period demonstrated that the most obvious effect this manipulation was the abolishment sexual dimorphism in the effect of prenatal BrdU treatment on striatal DA. This phenomenon suggests that female-specific hormones are necessary for the development of striatal DA function in females. Female sexual hormones seem to exert a protective effect on DA neurons. In adult rodents, less neurotoxicity of 6-OHDA on midbrain DA neurons [32] and of methamphetamine on striatal DA neurons have been reported in females compared with males [33]. Furthermore, it has been suggested that susceptibility of the striatal dopaminergic system to 6-OHDA is reduced in male rats but enhanced in female rats by gonadectomy [34]. A development study indicates that gonadal hormones in female mice during the pre-pubertal period are necessary for estrogen to exert neuroprotective effects on the nigrostriatal dopaminergic system [35]. In addition, adrenalectomy accompanied with oral corticosterone replacement reduces anxiety-like behavior in male rats but it does not have significant effects on females

[36]. A study of rhesus macaques has demonstrated that intact animals display less prepulse inhibition than animals given prepubertal castration [28]. This study also reveals that testosterone levels are correlated with tyrosine hydroxylase levels in the putamen among intact animals, suggesting the attenuation of PPI by gonadal sex hormones is mediated by dopaminergic activity in striatal regions. In addition, methamphetamine increases latent inhibition in male rats while this agent decreases this behavior in female rats, suggesting that presynaptic dopaminergic function shows a sex difference [37]. Hence, striatal DA function, especially presynaptic DA function may be different intrinsically between males and females.

The reduced DA and DOPAC in the striatum can be interpreted as a decreased total DA contents in presynaptic DA terminals that imply decrement of the number in DA presynaptic terminals or reduced DA synthesis rates. Ineffectiveness of DA receptor antagonists on hyperlocomotion found in BrdU-rats also supports abnormality in presynaptic function rather than postsynaptic DA receptors. In rodent, DA agonists usually facilitate locomotor activity. This study indicated opposite changes in striatal DA between males and females as an effect of prenatal BrdU while hyperlocomotion was obviously detected in both sex. Hence, the hyperlocomotion may be attributed to DA abnormality in the frontal cortex because the change was same in males and females.

Schizophrenia includes multiple pathology in brain functions. Striatal dysfunction is thought to be a fundamental element in schizophrenia [13]. A study using functional magnetic resonance imaging (fMRI) in schizophrenic patients has demonstrated that increased coherent intrinsic activity in the dorsal striatum during psychosis is predictive for delusion and hallucination and increased activity during psychotic remission in the ventral striatum is predictive for blunted affect and emotional withdrawal [38]. A positron emission tomography (PET) study has indicated an increased DA D₂/D₃ receptor density in a restricted area in the striatum [39]. A double-blind PET study has indicated that D₂ blockade in the striatum predicts antipsychotic response better than frontal, temporal, thalamic occupancy [40]. In addition, an involvement of the striatum in the cognitive impairment in schizophrenia has been proposed [41]. Furthermore, a study using recent molecular technique has shown that D₂ receptor overexpression in the striatum results in a functional deficit in the GABAergic system and this result suggests that the postulated deficit in GABAergic function in schizophrenia could be secondary to alterations in the striatum DA system [42].

It is hypothesized that psychosis is viewed as a process of aberrant salience [43] and a central role of DA is to mediate the salience of environmental events an internal representations [44]. A study using resting-state functional MRI has indicated increased that coherent intrinsic activity in the dorsal striatum during psychosis is predictive for delusion and hallucination, and that increased activity during psychotic remission in the ventral striatum is predictive for blunted affect and emotional withdrawal [38]. A meta-analysis of imaging studies using PET or single-photon emission computed tomography (SPECT) has indicated that the locus of the largest dopaminergic abnormality in schizophrenia is

presynaptic, which affects DA synthesis capacity, baseline synaptic DA levels, and DA release although a primary target of current antipsychotic drugs is blockade of DA D₂/D₃ receptors [45]. Higher DA concentration in the associative striatum in schizophrenia has been shown in a PET study using [¹¹C]raclopride, and this result suggests that elevated subcortical DA function adversely affect performance of the dorsolateral prefrontal cortex in patients [46]. ¹⁸F-dopa uptake into the associative striatum is elevated in patients with prodromal symptoms of schizophrenia. This finding using PET indicates that DA overactivity in individuals with prodromal psychotic symptoms [47]. This study also shows that striatal subdivision is negatively related to verbal fluency performance, but this is not the case for the limbic subdivision. Verbal fluency depends on prefrontal function [48]. The associative striatum regulates information flow to and from the prefrontal cortex [49, 50]. These findings provide a plausible mechanistic link between striatal dopaminergic dysfunction and prefrontal or executive dysfunction in schizophrenia. In addition, 5-HT_{2c} receptor antagonist increased incentive motivation in an animal model of the negative-symptoms of schizophrenia that was produced by increasing striatal-specific DA D₂ receptor density [51]. These data suggests a possibility that the primary focus of pathology of schizophrenia is the striatum, which includes abnormal presynaptic DA function, accompanied GABAergic and 5-HT dysfunction and parallel existence of aberrance in the prefrontal cortical function.

Although further investigation is needed, this BrdU-animal could be a possible animal model for schizophrenia given that it includes abnormal presynaptic striatal DA function with sexual dimorphism and frontal cortical dysfunction relating to DA hypersensitivity.

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