新生ラット視床下部における性ステロイド依存性遺伝子の発現解析

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Expression Analyses of Sex Steroid-Regulated Genes in Neonatal Rat Hypothalamus

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Abstract. Estrogen plays an important role in sexual differentiation of the brain in rats during the perinatal period. To elucidate molecular mechanisms underlying sexual differentiation of the brain, in this study we investigated genes differentially expressed between sexes or induced to express by estrogen in neonatal rat hypothalamus using DNA microarray analysis in combination with real-time RT-PCR. It was found that the levels of expression of the genes encoding glutamic acid decarboxylase 65 and coronin 1b were higher in male than female hypothalamus on postnatal day (PN) 5 and those of collagen type 3 α1 and thioredoxin reductase 2 genes in female hypothalamus on PN5 were decreased and increased, respectively, by treatment with estradiol on PN2. Then the developmental changes in the expression of these 4 genes were examined from 1 day before the parturition to PN9, and they all showed sexual dimorphic patterns. In addition, dependence of the expression of these genes on either estradiol, testosterone or dihydrotestosterone during the neonatal period was confirmed. These results suggest that these four genes are involved in sexual differentiation of the rat brain, and that androgen per se as well as estrogen may take part in the processes.

Key words: DNA microarray, Gene expression, Estrogen, Androgen, Sexual differentiation of brain

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Sexual differentiation of rat brain occurs during the perinatal period, i.e., around the day of birth to one week after birth, which is known as the critical period. In the male rat, testosterone secreted from the testis during the critical period induces the masculinization of the brain, while in the female, the absence of testosterone results in feminization of the brain [1, 2]. It has been demonstrated that aromatization of testosterone to estradiol in the brain plays an important role in mediating the effects of testosterone [3]. It is well known that estradiol affects many aspects of neuronal differentiation including apoptotic cell death [4], cell migration [5], synapse formation [6], and neurogenesis [7]. Most of these effects of estradiol occur through interactions with estrogen receptors, which serve as transcription factors for a wide variety of target genes [8]. To elucidate the molecular mechanism of sexual differentiation of the brain, it is important to study signaling cascades regulated by estradiol during the critical period. In this context, we have previously identified the granulin precursor gene as an estradiol- as well as testosterone-inducible gene in the rat hypothalamus during the critical period [9–11].

Testosterone can be converted to 5α-dihydrotestosterone (DHT), a non-aromatizable androgen, by 5α-reductase in the neonatal brain [12], but the significance of androgen or androgen receptors in the sexual differentiation of the brain is

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not well understood. Our previous studies have identified p130 as a gene the expression of which differs between sexes in neonatal rat hypothalamus [13], and further demonstrated that its expression is up-regulated by testosterone and DHT, but not estradiol [14], suggesting that androgen may be involved in the sexual differentiation of brain during the critical period by regulating the expression of genes such as p130.

In the present study, to further elucidate the molecular mechanisms underlying the sexual differentiation of rat brain, we first identified genes differentially expressed between sexes or induced to express by estrogen in the hypothalamus at postnatal day (PN) 5 by means of DNA microarray analysis in combination with real time RT-PCR. Then we examined the developmental expression of the genes in the hypothalamus during the perinatal period and the effects of estradiol, testosterone and DHT on the expression.

Materials and Methds

Animals and treatments

Wistar-Imamichi rats were housed in a temperature- and light-controlled room (23 ± 1 C; lights on 0500–1900 h) with food and water *ad libitum*. Pregnant females were prepared and allowed to deliver normally. At PN2, male pups were injected subcutaneously with 50 μl of vehicle (sesame oil) and females were injected with vehicle only or 10 μg estradiol benzoate (EB)/50 μl sesame oil. On PN5, pups were decapitated, the brain was immediately removed, and the entire hypothalamus including the preoptic area, bordered anteriorly by the optic chiasma, laterally by the hypothalamic fissures and posteriorly by the mammillary body, was dissected out [9]. Hypothalami were frozen in liquid nitrogen, and stored at –80 C until RNA isolation. To determine developmental gene expression patterns in the hypothalamus of intact male and female rats during the perinatal period, real-time RT-PCR was carried out on hypothalamic samples collected on PN-1 (one day before the parturition), PN1, PN3, PN5, PN7, and PN9. For the analysis of effects of sex steroids on gene expression, PN2 females were injected with either vehicle (sesame oil) only, or vehicle containing 10 μg of estradiol benzoate (EB), 100 μg of testosterone propionate (TP) or 800 μg of DHT, and decapitated on PN5.

DNA microarray analysis

DNA microarray analysis was done according to the manufacturer's protocol (Affymetrix GeneChip Expression Analysis Manual, Affymetrix Inc., Santa Clara, CA, USA) with slight modification. In brief, RNA was extracted from pooled tissue blocks obtained from 3 pups with TRIzol Total RNA Isolation Reagent (Life Technologies, Rockville, MD, USA), and the purity was checked by spectrophotometry and agarose gel electrophoresis. RNA was cleaned (Rneasy Mini Kit; Qiagen Inc., Valencia, CA, USA) and converted to double-stranded cDNA using an oligo dT primer containing the T7 promoter (5'- GGC CAG TGG AAT TGT AAT ACG ACT CAC TAT AGG GAG GCG G-dT_{24} -3') (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK) and SuperScript II (INVITROGEN). The cDNA was ethanol precipitated and used for preparing biotinylated cRNA (Bioarray High Yield RNA Transcription Labeling Kit; Enzo Diagnostics, Farmingdale, NY, USA). The biotinylated cRNA was cleaned (RNeasy spin columns; Qiagen Inc.), quantitated and fragmented, and the purity was checked by agarose gel electrophoresis. After confirmation of the quality of the fluorescent-labeled RNA target by hybridizing a small portion to the Affymetrix Test3 Array, 15 μg was hybridized to the Genechip Rat Genome U34A Array during 16 h of incubation at 45 C in an Affymetrix Fluidics Station 400. The chip was washed and stained with streptavidin-phycoerythrin. Each probe array was scanned twice with the probe array scanner and the data was analyzed with Microarrray Suite 4.0 software (Affymetrix).

Real-time PCR analysis

GeneChip results were confirmed by quantitative real-time PCR for 24 differentially expressed genes in each screening (data not shown). Total RNA was isolated from rat hypothalami using TRIzol reagent (Life Technologies Inc.) according to the manufacturer's instructions and cleaned (Rneasy Mini Kit; Qiagen Inc.). Total RNA (2 μl) from each sample was reverse-transcribed using the oligo $(dT)_{16}$ primer (Perkin-Elmer, Boston, MA, USA) and Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech Inc.) in a total volume of 33 μl

Table 1. PCR primers used in this study

Gene		Primer	Accession No.
GAD65	Sense	5′-GGA GCT GGC AGA GTA TTT A-3′	M72422
	Antisense	5′-CGG CTC ATT CTC TCT TCA T-3′	
corolb	Sense	5′-CCG GCA TGT ATT TGG TCA GCC AGT-3′	AJ006064
	Antisense	5′-TTG TGG GGG CAC CAG TCA ATA TCC-3′	
COL3A1	Sense	5′-TTG GAG GTG AAA AGT CTG GCG GCT-3′	M21354
	Antisense	5′-TGC AGC CTT GGT TAG GAT CAA CCC-3′	
TrxR2	Sense	5′-TGT CAA CGA GCA CAC AGT TCA CGG-3′	AF072865
	Antisense	5′-ACG TTT TCC CAG GGG ACT CCT TCA-3′	
RPS29	Sense	5′-TGA AGG CAA GAT GGG TCA CCA GCA GC-3′	X59051
	Antisense	5′-CAG GGT AGA CAG TTG GTT TCA TTG GG-3′	

GAD65, glutamic acid decarboxylase 65; corolb, coroninlb; COL3A1, collagen type 3 alpha 1; TrxR2, thioredoxin reductase 2; RPS29, ribosomal protein S29.

according to the manufacturer's instructions. For real-time PCR analysis, a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) was used. PCR primers used in this study are listed in Table 1. Dilutions (1:10, 1:100, 1:1000) of cDNA from each sample were used to construct a relative standard curve for each primer set. The linear relationships between the log concentration of template and cycle number were obtained ($r = -1$, error < 0.02 for all genes). The amplification program consisted of 1 cycle of 95 C with a 10 min hold (hot start) followed by 40 cycles of 95 C with a 15 sec hold, 65 C with a 10 sec hold, and 72 C with a 14 sec hold. Acquisition was done at the end of a 14 sec hold at 72 C. Amplification was followed by melting curve analysis. Both non-reverse transcribed samples and water as a negative control were run to assess PCR specificity for constructing standard curves. The level of mRNA expression was normalized to RPS29 because it is considered a stable housekeeping gene and was detected at the same level in each sample used.

Stastical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) analysis. Significance was defined as $P<0.05$.

Results

Twenty-four genes whose expression was different between the sexes and 24 genes whose expression was induced by EB, as assessed by DNA microarray analysis (data not shown), were further quantitatively analyzed by real-time PCR. Among these genes, we identified 4 genes whose expression pattern was consistent by both DNA microarray and real-time PCR analyses. As shown in Fig. 1, the expression levels of the glutamic acid decarboxylase 65 (GAD65) and coronin 1b (corolb) genes were significantly higher in male than female hypothalamus on PN5, and the expression of the collagen type 3 α1 (COL3A1) and thioredoxin reductase 2 (TrxR2) genes in female hypothalamus on PN5 was significantly decreased and increased,

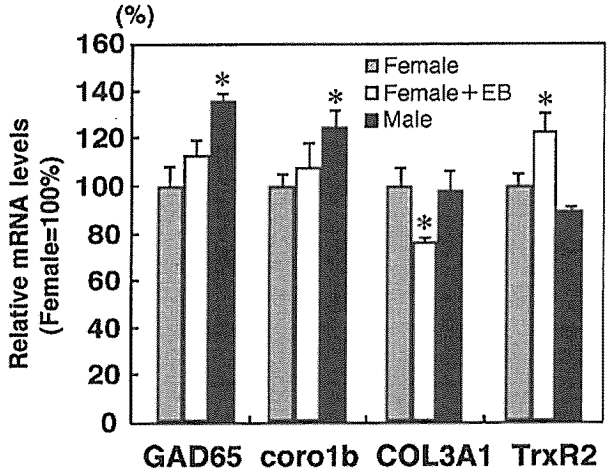

Fig. 1. Effects of sex and EB on gene expression in PN5 rat hypothalamus analyzed by real-time RT-PCR. The levels of gene expression were measured 72 h after EB treatment in PN2 females. A significant sex difference was seen for GAD65 and coro1b, and a significant effect of EB was seen for COL3A1 and TrxR2 (* $P<0.05$ vs. female). Each value is normalized using RPS29. Female and male groups were injected with sesame oil. Each column and vertical bar represents the mean ± SEM (n=4–5/group).

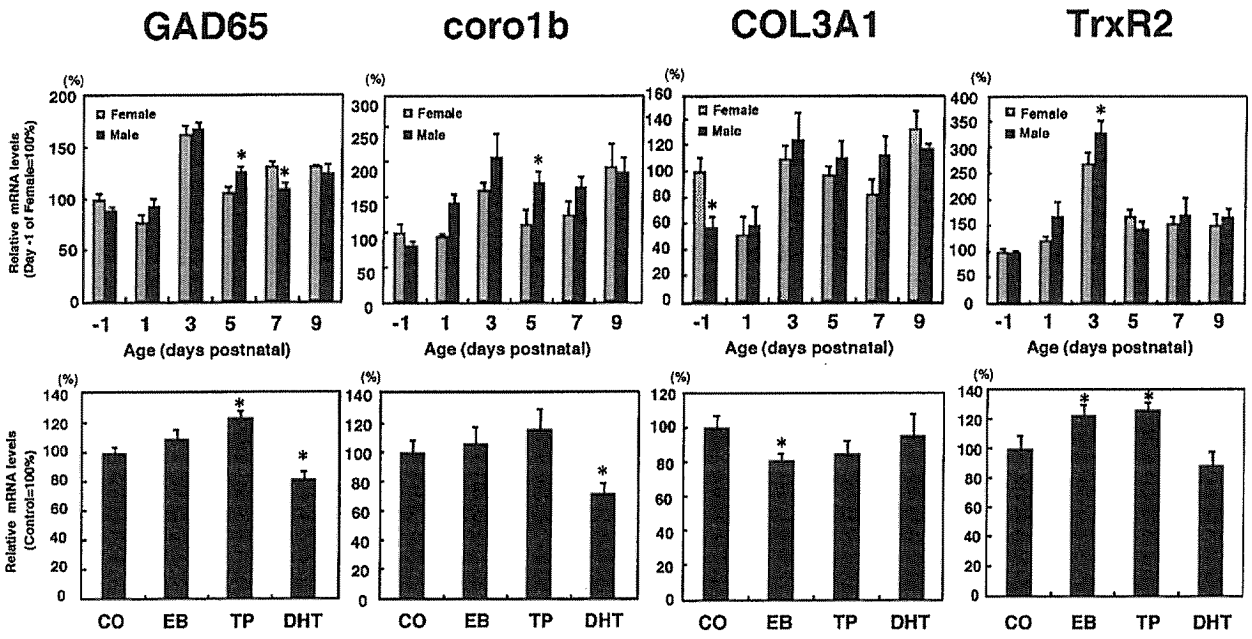

Fig. 2. Gene expression in rat hypothalamus analyzed by real-time RT-PCR. Upper panels: Developmental expression in the male and female (n=3–9/group). *, P<0.05, male vs. female. Lower panels: Effects of EB, TP and DHT on gene expression in the female hypothalamus (n=3–14/group). *, P<0.05 vs. control (CO). The levels of gene expression were measured at 72 h after steroid treatment in PN2 females. CO groups were injected with sesame oil. Each value is normalized using RPS29. Each column and vertical bar represents the mean ± SEM.

respectively, by EB treatment on PN2.

We then analyzed developmental expression of these 4 genes in the hypothalamus of intact male and female rats during the perinatal period using real-time RT-PCR (Fig. 2, upper panels). A post-hoc Fisher's PLSD analysis indicated that, at PN5, GAD65 mRNA expression was significantly greater in males than females, but at PN7 that in males was less than that in females. As for coro1b, males at PN5 had a significantly elevated mRNA levels compared to females at PN5. Expression level of COL3A1 mRNA was significantly greater in females than males at PN-1, while there was no significant difference in the gene expression after birth. TrxR2 mRNA expression was significantly greater in males than females at PN3.

Finally, we analyzed the effects of EB, TP and DHT treatment at PN2 on the expression of these four genes in female hypothalamus at PN5 (Fig. 2, lower panels). TP significantly increased the expression of GAD65 mRNA, whereas DHT decreased it. Coro1b mRNA expression was down-regulated by DHT. The expression of COL3A1 mRNA was down-regulated by EB, but not TP and DHT. The expression of TrxR2 mRNA was up-regulated by EB and TP, but not DHT.

Discussion

Using DNA microarray and real-time RT-PCR, we identified two genes the expression of which differs between sexes and two EB-regulated genes in neonatal rat hypothalamus. Further analysis using real-time RT-PCR revealed that the expression of genes identified as male-enriched, GAD65 and coro1b, was androgen-responsive. We also revealed that the expression of two EB-regulated genes, COL3A1 and TrxR2, showed sex differences in the perinatal period in intact animals. These results suggest that the expression of genes differentially expressed between sexes in perinatal rat hypothalamus is primarily regulated by sex steroids.

GAD65 is a rate-limiting enzyme in the synthesis of GABA. McCarthy *et al.* [15] have shown that sex differences in the GABAergic system during the neonatal period may contribute to the sexual differentiation of the rat brain. They showed that expression of GAD65 mRNA in the dorsomedial

hypothalamic nucleus was increased in males and was induced by TP treatment [16]. In adult rat hypothalamus and the preoptic area, the expression of GAD65 mRNA is regulated by estrogen [17]. Using the Genbank database (AF090195), we identified seven estrogen responsive element (ERE)-half sites (5′-GGTCA-3′ or 5′-TGACC-3′) and an imperfect palindromic ERE (5′-AGGTCACAGCGACCT-3′) in the 5′-flanking region (from –2000 to –1) of the gene. However, we did not find any androgen responsive elements in the promoter. Thus, although we found that the expression of GAD65 mRNA was affected by androgen, but not by estrogen, the effect of androgen on GAD65 gene expression might be indirect.

Coro1b is one of several coronin-like proteins identified in many species [18]. So far, no study has reported the function or expression site of this gene. The coronin-like protein in yeast promotes actin polymerization and also interacts with microtubules [18]. The higher expression level of coro1b in male than female hypothalamus during the critical period suggests that coro1b participates in the remodeling of the neuronal and/or glial cytoskeleton, which is thought to be an important event for constructing a sex-specific neural circuit.

Of the identified estrogen target genes, the COL3A1 gene has been described as estradiol-regulated. COL3A1 is a major component of vascular matrix and its expression in cultured bovine aortic smooth muscle is down-regulated by estradiol treatment [19]. Interestingly, the composition of the vascular matrix is closely related to the progression of diseases such as atherosclerosis and restenosis [20]. Since it has been demonstrated that estrogen has beneficial cerebrovascular effects, causing vasodilation or anti-atherogenesis [21], it is possible that COL3A1 mediates the cerebrovascular effect of estrogen.

TrxR2 belongs to a superfamily of flavoprotein disulfide oxidoreductases that includes glutathione reductase. TrxR2 is localized to mitochondria, providing a primary line of defense against H_2O_2 produced by the mitochondria respiratory chain [22]. Estradiol has antioxidant neuroprotective activity, though its mechanism of action remains largely unknown [23]. TrxR2 might mediate the antioxidant effect of estradiol, which is produced via aromatization of testosterone, on neurons and is involved in the regulation of cell survival, which is an important cellular event involved in sexual differentiation of the brain.

It seemed paradoxical that the gene expression of GAD65 and coro1b was elevated in PN5 males, but the expression of these genes in females was down-regulated by DHT treatment. It was demonstrated that androgens exert a biphasic dose-dependent effect on E2F-1 expression levels [24]. It is therefore possible that treatment with different doses of DHT results in the up-regulation of GAD65 and coro1b expression. In addition to these genes, our previous study identified a DHT-responsive gene, p130, in neonatal rat hypothalamus [14]. Considering that DHT is an androgen that cannot be aromatized, we suggest that the activation of AR affects the development of the brain during the critical period for sexual differentiation by regulating the expression of its target genes.

In conclusion, we identified four genes the expression of which is estrogen- and/or androgen-responsive and differs between sexes in rat hypothalamus during the critical period for sexual differentiation of the brain using DNA microarray screening in combination with real-time RT-PCR. The identification of these genes enhances our understanding of the sex steroid regulatory cascade in developing brain and may help to elucidate the molecular mechanisms underlying sexual differentiation of the brain.

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