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ORIGINAL PAPER

Developmental exposure to pentachlorophenol affects the expression of *thyroid hormone receptor β1* and *synapsin I* in brain, resulting in thyroid function vulnerability in rats

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Abstract Pentachlorophenol (PCP), a component of biocides and a contaminant in diverse tissue samples from humans from various geographic areas, disrupts regulatory effects of thyroid hormones. Here we examined the effects of developmental exposure of rats to PCP on various aspects of brain development, male reproductive function, and adrenal function, all of which are under thyroid hormones regulation. PCP was administered to dams and their offspring via drinking water (6.6 mg l^{-1}) during gestation and lactation. Tissue samples were obtained from dams, 3-week-old weanling pups, and 12-week-old pups. Gene expressions of *thyroid hormone receptor β1* and *synapsin I*, factors that promote brain growth, was increased in the cerebral cortex of PCP-treated weanling females, whereas plasma concentrations of total thyroxine were decreased in dams and weanling pups, and plasma thyroid-stimulating hormone concentrations were higher in PCP-treated weanling males. PCP caused a decrease in plasma corticosterone concentrations in 12-week-old female rats, but not in male rats or weanling females. PCP-treated male pups had significantly increased testis weight at 12 week of age. No overt signs of toxicity were noted throughout this study. Our results show that PCP exposure during development causes thyroid function vulnerability, testicular hypertrophy in adults, and aberrations of brain gene expression.

Keywords Pentachlorophenol · Development · Thyroid hormone · Cerebral cortex · Female · Rat

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Introduction

Pentachlorophenol (PCP), a halogenated phenolic compound used in wood preservatives, biocides, and disinfectants, disrupts thyroid hormones function in rats [1], ewes [2], and cell lines [3, 4]. Other halogenated phenolic compounds, such as polychlorinated biphenyls (PCBs), are well known to induce developmental abnormalities due to disruption of thyroid grand function in mice, rats, chickens, humans, and cultured cells [5–8]. In Quebec, concentrations of thyroxine (T4) and triiodothyronine (T3) were decreased in plasma from human umbilical cord blood depending on the amount of PCP present, but PCBs had no effect on the amounts of these hormones [9]. In addition, PCP has appeared as a contaminant in samples of plasma [10–19], cerebrospinal fluid [20], umbilical cord blood [9, 10, 14], amniotic fluid [21], and breast milk [14, 22–24] of humans from various geographic areas. These findings suggest that PCP may alter thyroid gland status in newborns, which in turn could cause developmental abnormalities, especially of the brain. Nevertheless, whether PCP exposure during development affects brain growth is unknown. Moreover, a limited number of reports have detailed the effects of PCP exposure during development on disruption of thyroid hormones [25].

Thyroid hormones perform various functions that are essential for development. For example, hypothyroidism in developing rats results in abnormal brain development, characterized by diminished interneuronal connectivity and altered neurotransmitter release [26–30]. Many genes involved in thyroid hormone regulation of brain development are known. Physiological actions of thyroid hormone are usually mediated through interaction with nuclear receptors, and *thyroid hormone receptor β1* (*TRβ1*), one of the thyroid hormone receptor isoforms. *Neurogranin* (*RC3*) and *synapsin I* are implicated in dendritic spine function, neurotransmitter release, and learning [27, 31–35]. Moreover, neonatal hypothyroidism results in increased sperm production and testicular size in adults, because thyroid hormones inhibit proliferation of neonatal Sertoli cells [36–38]. In addition, neonatal hypothyroidism impairs the developmental rise in circulating corticosterone [39, 40] and delays maturation of the hypothalamic–pituitary–adrenal axis response to stressors [41, 42].

In this study, we analyzed the effects of developmental PCP treatment on thyroid hormones-regulated aspects of the male reproductive system, adrenal function, and brain development associated with expression of the cerebral cortex genes for *TRβ1*, *RC3*, and *synapsin I* in rats.

Results

No overt signs of toxicity were noted in this study (Table 1). There were no observed effects of PCP exposure on litter size (control litter; 15.4 ± 1.7, PCP-treated litter; 14.0 ± 2.5).

Expression of cerebral cortex genes

After exposure of their dams to PCP throughout gestation and lactation, brains collected from 3-week-old weanling pups were processed for reverse transcription-polymerase chain reaction (RT-PCR) analysis to investigate the effects of the chemicals on the expression of various genes on the cerebral cortex. Levels of *TRβ1* and *synapsin I* transcripts in PCP-treated 3-week-old female rats increased significantly ($P < 0.05$) to about 3 and 2.5 times that of the control group, respectively (Fig. 1a, c). The expression of *RC3* prepared from PCP-treated 3-week-old female rats was about 2.5 times that of the control (Fig. 1b), but this change was not statistically significant. These gains in gene expression did not occur in 12-week-old female rats (Fig. 1d–f), and transcript levels of all genes that we evaluated were similar between PCP-treated and control male rats (Fig. 2). There was no change in an endogenous control gene, *18S rRNA* levels, between PCP-treated and control in all groups.

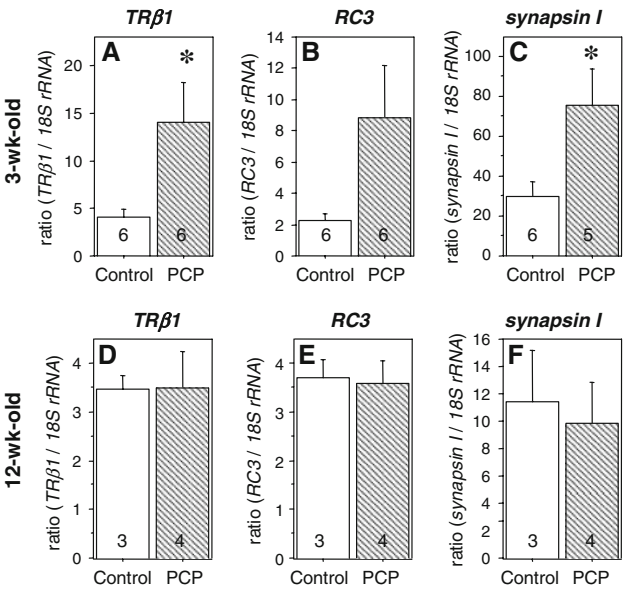

Fig. 1 Effects of developmental exposure to PCP on mRNA expression of *thyroid hormone receptor β1* (*TRβ1*) **a**, **d**, *neurogranin* (*RC3*) **b**, **e**, and *synapsin I* **c**, **f** in the frontal cortex of 3-week-old **a–c** and 12-week-old **d–f** female rats. PCP treatment induced expression of the genes for *TRβ1* and *synapsin I* in 3-week-old female pups. The relative intensity of gene expression was quantified by a standard method according to the manufacturer's protocol and normalized to *18S rRNA*. Values are shown as mean ± SEM; numerals in bars indicate the number of rats in each group. *$P < 0.05$ compared with control value when analyzed by using the Mann–Whitney *U*-test or Student *t*-test

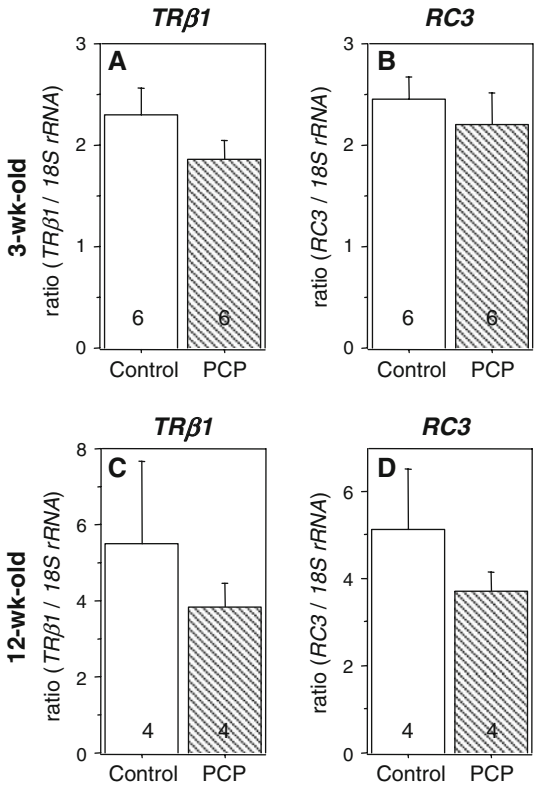

Fig. 2 Effects of developmental exposure to PCP on mRNA expression of the genes for *TRβ1* (**a**, **c**) and *RC3* (**b**, **d**) in the frontal cortex of 3-week-old (**a**, **b**) and 12-week-old (**c**, **d**) male rats. The relative intensity of gene expression was quantified by a standard method according to the manufacturer's protocol and normalized to *18S rRNA*. Values shown are mean ± SEM; numerals in bars indicate the number of rats in each group

Thyroid function

Plasma concentrations of total thyroxine (TT4) were significantly ($P < 0.05$; Mann–Whitney *U*-test) decreased in PCP-treated dams (Table 2). Although plasma concentrations of thyroid-stimulating hormone (TSH) also tended to decrease in PCP-treated dams, the differences did not achieve statistical significance.

Compared with concentrations in the controls, plasma concentrations of TSH were significantly higher ($P < 0.05$; Table 4) in PCP-treated 3-week-old male pups, whereas TT4 levels tended to decrease, albeit not significantly. Plasma levels of total triiodothyronine (TT3) did not differ between control and PCP-treated 3-week-old male rats. In 12-week-old male rats, plasma concentrations of TT3 and TT4 tended to decrease than those in controls, but differences did not achieve statistical significance. Plasma concentrations of TT4 tended to be lower in 3- and 12-week-old female pups than in their controls (Table 5). Two-way analysis of variance (ANOVA) revealed significant effects of treatment but not gender in the TT4 levels of 3-week-old pups ($P < 0.05$). Therefore, given the lack of a gender effect on these data, exposure to PCP during development significantly decreased plasma TT4 in weanling pups.

Table 1 Body weight and organ weight in dams after exposure to pentacholorophenol (PCP) during gestation and lactation

Treatment	Body weight (g)	Liver weight (g)	Adrenal weight (mg)
Control	340 ± 9 (6)	17.2 ± 0.8 (6)	32.5 ± 1.5 (6)
PCP	340 ± 5 (7)	16.9 ± 0.3 (7)	31.5 ± 1.2 (7)

Results are expressed as mean ± SEM. Numerals in parentheses indicate the number of rats in each group

Table 2 Plasma hormone concentrations in dams after exposure to PCP during gestation and lactation

Hormone	Control	PCP
TT4, μg dl^{-1}	2.84 ± 0.24 (6)	1.72 ± 0.20 (7)*
TT3, ng dl^{-1}	67.37 ± 1.15 (6)	67.91 ± 6.68 (7)
TSH, pg ml^{-1}	1.02 ± 0.36 (6)	0.52 ± 0.23 (7)
Corticosterone, ng ml^{-1}	851 ± 168 (6)	1112 ± 123 (7)

Results are expressed as mean ± SEM. Numerals in parentheses indicate the number of rats in each group

* $P < 0.05$ (Mann–Whitney *U*-test) compared with value for control group

Adrenal function

Neither adrenal weight nor plasma corticosterone levels differed significantly between control and PCP-treated dams (Tables 1, 2) and pups (Tables 3–5), except that plasma corticosterone decreased in 12-week-old female rats ($P < 0.05$; Table 5).

Male reproduction

We evaluated the effect of neonatal PCP treatment on markers of reproductive function in adult male rats. Absolute (Table 6) and relative (data not shown) testis weights were significantly increased in 12-week-old PCP-treated male pups compared with controls ($P < 0.05$). However, plasma concentrations of ir-inhibin, testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) and sperm number did not differ between control and PCP-treated male rats (Table 6).

Discussion

Developmental exposure to PCP caused pronounced increases in the expression of *TRβ1* and *synapsin I* mRNA in the cerebral cortex of PCP-treated 3-week-old female rats. The expression of *RC3* tended to lower in PCP-treated 3-week-old

Table 3 Body weight and organ weight in pups after exposure to PCP during gestation and lactation

Treatment	3-week-old pups		12-week-old pups	
	Male	Female	Male	Female
Body weight (g)				
Control	43.9 ± 2.5 (6)	41.1 ± 3.1 (6)	456 ± 13 (6)	276 ± 7 (6)
PCP	41.7 ± 1.7 (7)	37.9 ± 2.3 (7)	433 ± 14 (7)	261 ± 6 (7)
Liver weight (g)				
Control	2.31 ± 0.54 (6)	2.31 ± 0.17 (6)	20.7 ± 0.7 (6)	13.2 ± 0.5 (6)
PCP	1.69 ± 0.14 (6)	1.53 ± 0.16 (7)	19.3 ± 0.8 (7)	12.5 ± 0.3 (7)
Adrenal weight (mg)				
Control	6.1 ± 0.8 (6)	5.7 ± 0.3 (6)	31.2 ± 1.7 (6)	36.7 ± 0.9 (6)
PCP	6.2 ± 0.4 (7)	5.4 ± 0.6 (7)	30.6 ± 0.6 (7)	35.6 ± 2.1 (6)

Results are expressed as mean ± SEM. Numerals in parentheses indicate the number of rats in each group

Table 4 Plasma hormone concentrations in male pups after exposure to PCP during gestation and lactation

Hormone	3-week-old	12-week-old
TT4, μg dl^{-1}		
Control	3.87 ± 0.51 (5)	3.56 ± 0.27 (5)
PCP	3.16 ± 0.27 (5)[a]	3.20 ± 0.11 (7)
TT3, ng dl^{-1}		
Control	77.0 ± 19.2 (5)	99.5 ± 6.4 (5)
PCP	103.1 ± 2.6 (3)	88.4 ± 6.3 (7)
TSH, pg ml^{-1}		
Control	0.45 ± 0.07 (5)	1.42 ± 0.68 (6)
PCP	0.70 ± 0.09 (6)*	0.88 ± 0.23 (7)
Corticosterone, ng ml^{-1}		
Control	451 ± 71 (6)	99 ± 16 (5)
PCP	416 ± 62 (6)	172 ± 51 (7)

Results are expressed as mean ± SEM. Numerals in parentheses indicate the number of rats in each group

* $P < 0.05$ (Mann–Whitney *U*-test) compared with value for control group

[a] Convine with female, two-way analysis of variance revealed significant effects of treatment but not gender in the TT4 levels of 3-week-old pups ($P < 0.05$)

Table 5 Plasma hormone concentrations in female pups after exposure to PCP during gestation and lactation

Hormone	3-week-old	12-week-old
TT4, μg dl^{-1}		
Control	3.75 ± 0.31 (6)	3.51 ± 0.28 (6)
PCP	3.00 ± 0.22 (7)[a]	3.06 ± 0.23 (7)
TT3, ng dl^{-1}		
Control	Not evaluated	93.0 ± 6.2 (6)
PCP	Not evaluated	87.0 ± 3.6 (7)
TSH, pg ml^{-1}		
Control	Not evaluated	0.50 ± 0.07 (6)
PCP	Not evaluated	1.07 ± 0.37 (7)
Corticosterone, ng ml^{-1}		
Control	437 ± 31 (6)	289 ± 89 (6)
PCP	386 ± 40 (7)	103 ± 26 (7)*

Results are expressed as mean ± SEM. Numerals in parentheses indicate the number of rats in each group

* $P < 0.05$ (Mann–Whitney *U* test) compared with value for control group

[a] Convine with male, two-way analysis of variance revealed significant effects of treatment but not gender in the TT4 levels of 3-week-old pups ($P < 0.05$)

female rats, but did not achieve statistical significance. This is the first report of the effects of PCP on gene expression in the rat brain, and this result may reflect thyroid hormones disruption. The physiological effects of thyroid hormones usually are mediated through interaction with nuclear receptors, and TRβ1 is one isoform of the thyroid hormone receptor. In previous reports, neonatal (age, 2–3 weeks) hypothyroid rats show increased expression of synapsin I [27]; conversely, T3 and T4 induce the expression of synapsin I in cortical cultures [43, 44]. In our study, PCP exposure significantly reduced plasma levels of TT4 in dams and 3-week-old pups. Hence, the increased levels of *synapsin I* noted in with PCP treatment may be attributable to the decreased levels of serum T4 or to the increased expression of *TRβ1*. In contrast, PCP increased cellular uptake of T3 in vitro by displacing T3 from its plasma binding sites [45]. Therefore, the higher levels of gene expression seen after PCP treatment may be attributable to increased cellular uptake of T3. In other hypothesis, PCP treatment may increase conversion of T4 to T3 by type II deiodinases, or may increase expression and function of cellular hormone transporters.

Synapsin I is implicated in synaptic plasticity, neurotransmitter release, and learning [39, 40, 46]. Electrophysiology studies suggest that synapsin I contributes to the action of thyroid hormones on paired-pulse facilitation and neurotransmitter release [27]. Consequently, developmental exposure to PCP may affect synaptic plasticity and neurotransmitter release. In contrast, PCP did not affect the expression of *TRβ1* or *synapsin I* in 12-week-old female rats, perhaps because these animals had not received PCP since

Table 6 Testis weight, sperm number, and plasma hormone concentrations in 12-week-old male pups after exposure to PCP during gestation and lactation

	Control	PCP
Testis weight, g	2.73 ± 0.05 (6)	2.89 ± 0.05 (7)*
Sperm number, $\times 10^7$	5.94 ± 1.05 (5)	6.04 ± 1.41 (7)
ir-Inhibin, pg ml^{-1}	0.98 ± 0.19 (5)	1.04 ± 0.18 (7)
Testosterone, ng ml^{-1}	11.3 ± 1.8 (6)	16.1 ± 2.4 (7)
Luteinizing hormone, pg ml^{-1}	737 ± 128 (6)	1123 ± 339 (6)
Follicle-stimulating hormone, pg ml^{-1}	7.08 ± 0.85 (6)	8.11 ± 0.74 (7)

Results are expressed as mean ± SEM. Numerals in parentheses indicate the number of rats in each group

* $P < 0.05$ (Mann–Whitney *U*-test) compared with value for control group

they were weaned on 3-week-old and, consequently, the burden of PCP on the rat's body would have diminished due to metabolic clearance. Sui et al. have shown that persistent effects of moderate developmental hypothyroidism on adult hippocampal synaptic functions include decreased paired-pulse facilitation and increased magnitude of long-term potentiation of somatic population spike, without changes in synaptic proteins such as synapsin and RC3 [47]. The decrease in paired-pulse facilitation due to the increased levels of synapsin protein in neonates with hypothyroidism [27] may contribute to the persistent behavioral deficits associated with developmental hypothyroidism [47]. Perhaps the neonatal PCP treatment in the present study would induce similar persistent electrophysiological and behavioral changes.

In contrast to the findings from female rats, developmental exposure to PCP did not affect expression of the examined genes in 3-week-old male rats, despite the lack of a gender-associated difference in plasma TT4. Estrogen and thyroid hormone receptors interact [48], and thyroid hormone receptor isoforms stimulates or inhibited estrogen receptor-mediated transcription [49, 50]. On the other hand, estrogen induces the production of synaptic proteins, such as synapsin and RC3 [51–53]. Hence, reduction of thyroid hormones effectiveness due to developmental exposure to PCP may accelerate the estrogen-stimulated mRNA expression of *synapsin I* and *RC3* in female rats. Only a few studies have investigated the sex-associated differences in thyroid hormones-induced gene expression in the brain. Recently, Dong et al. identified global gender-associated differences in gene expression in the developing cerebellum in response to hypothyroidism, although the mechanisms underlying these differences are unclear [54]. Future studies will reveal the mechanisms modulating gender-associated differential susceptibility to PCP and thyroid hormones in the brain.

Our results confirmed that PCP exposure significantly reduced plasma levels of TT4, and tended to reduce plasma levels of TSH in dams. This observation has been documented amply in adult rats [1, 55], and PCP may decrease thyroid hormones and TSH levels through interference at the pituitary or hypothalamic level. In addition, developmental exposure to PCP significantly reduced plasma levels of TT4 in 3-week-old pups. This finding is in agreement with other studies in which maternal exposure to PCP decreased circulating T4 levels in rams [25] and in which concentrations of T3 and T4 were decreased in plasma from human umbilical cord blood depending on the amount of PCP present in Quebec [9]. In the present study, pups were exposed to PCP in utero and then through their dams' milk just before weaning, they might also have consumed PCP-treated water. The decreased T4 levels in the weanling pups may reflect accommodation of the thyroid system to the effects of PCP or a reduction in the T4 levels of the dams. Further, PCP exposure significantly induced higher plasma TSH levels in the weanling male rats, possibly reflecting amplification of negative feedback.

Developmental PCP treatment increased testicular weight in our adult male rats. In maternal PCP-treated rams (1 mg kg^{-1} day^{-1}), T4 levels were suppressed, and focal degenerative changes were present in the seminiferous tubules of testis, leading to a significant increase in the severity of seminiferous tubule atrophy compared with that in controls [25]. In addition, PCP-treated rams had heavier testes than did untreated rams, although this difference was not statistically significant due to marked interanimal variation. Therefore, PCP may exert these effects on the testis by inducing hypothyroidism, which leads to increased Sertoli cell proliferation and testis weight. Similarly, neonatal PCB treatment apparently induces hypothyroidism and increases adult testis size and sperm production [56]. In our study, however, developmental PCP treatment did not affect plasma ir-inhibin, plasma testosterone, plasma LH, plasma FSH, or sperm production. In contrast, neonatal high-dose PCP treatment (30 or 60 mg kg^{-1} day^{-1}) led to small testes, decreased spermatid count, and delayed sexual maturation in rats [57]. However, this reproductive disability in animals given high-dose PCP may be attributable to toxicity, in light of concurrent induced liver weight with histopathologic toxicity, reduced body weight, and reduced feed consumption.

Markers of adrenal function did not differ between our control and PCP-treated rats, except for increased plasma corticosterone in 12-week-old female rats. Although hypothyroidism impairs the hypothalamic–pituitary–adrenal axis [58–60], PCP may not have altered adrenal function in the present study. Exposure to PCP altered plasma corticosterone in 12-week-old female rats despite the lack of a gender-associated difference in thyroid hormones. Adrenal function typically is more sensitive in female animals than in males [61, 62]. The explanation for this difference may involve changes in plasma estradiol levels, because estrogen induces basal and stress-

responsive corticosterone levels in ovariectomized rats [63], and infusion of estrogen antagonist into the paraventricular nucleus inhibited corticosterone levels associated with the stress response in rats [64]. However, maternal PCP treatment did not change plasma estradiol levels in 12-week-old females (data not shown).

In summary, the present study shows that PCP exposure during gestation and lactation causes moderate thyroid function vulnerability, adult testicular hypertrophy, and aberrations in gene expression in the cortex. These results are the first that suggest that PCP may affect growth of the brain. Even mild perturbations in thyroid hormonal status in pregnant women and animals have been associated with long-lasting impairment, including impaired brain function, in their offspring [47, 65–68]. In addition, PCP exposure during development may aggravate persistent hypofunction in animal and human brains.

Materials and methods

Animals and treatment

Wistar–Imamichi rats were obtained from the Institute for Animal Reproduction (Ibaraki, Japan) and maintained under conditions of controlled lighting (lights on, 07.00–19.00), temperature (22.5 ± 0.5°C), and humidity (55% ± 10%). Food (CE2, CLEA Japan, Inc., Tokyo, Japan) and distilled water in glass bottles were available ad libitum. Adult rats and nursing dams with pups were housed at one adult per stainless cage lined with paper bedding (Paper Clean, Japan SLC, Inc., Shizuoka, Japan). Animals were maintained and used according to the guidelines of the National Institute for Environmental Studies Animal Care and Use Committee.

Exposure schedule

Thirteen-week-old virgin female rats were paired with 10-week-old fertile male rats from the evening of proestrus to the morning of estrus. The stage of the estrous cycle was determined based on vaginal cytology. Female rats that showed a regular 4-day estrous cycle 2 weeks prior to mating were used in the present study. Mating was verified by the presence of sperm in vaginal smears (gestational day 0; GD0), when females were separated from the males. PCP was administered to dams and offspring via drinking water (6.6 mg l^{-1}) from GD0 to postnatal day (PD) 21 (PD0 = day of birth). Water intake was measured during PCP treatment. The oral route of administration was selected, because it is a potential route of human exposure and has the advantage of avoiding the stress associated with injection or gavage. The total number of pregnant animals in control and PCP-treated group were 6 and 7, respectively. Pups were counted and culled on PD3 to four males and four females per litter and kept with their respective dams until weaning on PD21.

Sample collection

Dams were weighed and decapitated at weaning. One male and one female pup per litter were weighed and decapitated each at 3 and 12 weeks of age; 12-week-old female pups were in proestrus. After decapitation, trunk blood, liver, adrenal gland, testis (12-week-old male rats only), and brain (pups only) were collected, and organs other than the brain were weighed. The right testis was decapsulated, placed in a measured volume of saline, and frozen at −20°C for the measurement of total sperm number. All blood samples were collected into plastic tubes containing heparin to prevent clotting. The samples were stored in ice and centrifuged at 3000 × *g* for 10 min at 4°C immediately after completing the experiment. The resulting plasma was stored at −20°C for analysis of TT3, TT4, TSH, LH, FSH, testosterone, ir-inhibin, estradiol, and corticosterone levels. Brains were placed immediately on dry ice and stored at −80°C for mRNA analysis of *TRβ1*, *RC3*, and *synapsin I* (female pups only).

Extraction of mRNA, cDNA synthesis, and RT-PCR

Total RNA was extracted from the frontal cortex by using the RNeasy Lipid Tissue Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

For each sample, 2 μg RNA was reverse-transcribed in a 40-μl reaction medium of 1 mM dNTPs, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 μM random hexamer, 1 unit μl^{-1} RNase inhibitor, and 1 unit μl^{-1} MuLV reverse transcriptase (Perkin–Elmer, Foster City, CA, USA).

Reverse transcription was performed at 42°C for 15 min and 99°C for 5 min. The mixture was then cooled to 5°C and stored at −20°C until use. The quantification of mRNA expression was performed using the ABI Prism 7000 Sequence Detection System (Perkin–Elmer) according to the manufacturer's instructions. Oligonucleotide hybridization probes (TaqMan probes) were labeled with a 5′ fluorescent reporter dye (6FAM or VIC) and a 3′ quencher dye (TAMRA). The 5′-to-3′ nuclease activity of *Taq* DNA polymerase cleaved the probe and released the reporter, whose fluorescence was detected by the laser detector of the ABI Prism 7000. The increase in fluorescence was monitored throughout amplification. The quantification of gene expression was derived from the cycle number at which the fluorescent signal crossed a threshold during the exponential phase of the PCR reaction, using the standard curve method according to the manufacturer's protocol.

The fluorescence intensity of each sample was normalized to that of an endogenous control gene (*18S rRNA*). PCR amplification was performed in a 20-µl final reaction mixture consisting of 10 µl 2× TaqMan Universal PCR Master mix (Perkin–Elmer), 2 µl 20× Assay mix (supplied with TaqMan probe and pair primers), and 1 µl cDNA. cDNAs were amplified according to the thermal profile of 50°C for 2 min then 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. PCR primer pairs and the TaqMan probe were purchased from Perkin–Elmer. The sequences of *TRβ1*, *RC3*, *synapsin I*, and *18S rRNA*, which we purchased from Perkin–Elmer, were not disclosed by the manufacturer.

Measurement of plasma hormone levels

Plasma was analyzed for rat TSH, LH, and FSH by radioimmunoassay by using the rat kit from the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK; Bethesda, MD, USA). Iodinated preparations were rat-TSH-I-9, rat-LH-I-7, and rat-FSH-I-7; antisera used were anti-rat TSH-S-5, anti-rat LH-S-10, and anti-rat FSH-S-11. Results were expressed as rat TSH RP-2, rat LH RP-2, and rat FSH RP-2. The intra-assay coefficients of variation (CVs) were 4.8% for TSH, 8.9% for LH, and 4.4% for FSH. The plasma concentrations of TSH, LH, and FSH were measured in a single assay.

The plasma concentrations of ir-inhibins were measured using a rabbit antiserum against purified bovine inhibin (TNDH 1) and ^{125}I-labeled 32-kDa bovine inhibin, as described previously [69]. Estradiol were measured by double-antibody RIA using ^{125}I-labeled radioligands as described previously [70]. Antisera against estradiol (GDN 244; [71]) were kindly provided by Dr. G.D. Niswender (Colorado State University, Fort Collins, CO, USA). The intra-assay CVs were 4.1% for estradiol and 8.8% for ir-inhibin. The plasma concentrations of estradiol and ir-inhibin were measured in a single assay.

Plasma TT3, TT4, testosterone, and corticosterone were determined by radioimmunoassay by using commercial kits (Diagnostic Products, Los Angeles, CA, USA). The intra-assay CVs were 3.7% for TT3, 2.7% for TT4, 6.3% for testosterone, and 9.8% for corticosterone. The plasma concentrations of TT3, TT4, testosterone, and corticosterone were measured in a single assay.

Sperm production

Changes in spermatogenesis in the testis were evaluated as described previously [72]. In brief, one drop of extracted emulsion was placed on a hematocytometer after sufficient dilution with saline, and sperm heads then were counted under a phase-contrast microscope.

Statistical analyses

All results are expressed as mean ± SEM. When data from only two groups were available, the Mann–Whitney *U*-test was applied. If the Mann–Whitney *U*-test was not significant, the Student *t*-test was applied after confirming the equality of variance by the *F*-test for two samples. For multiple comparisons, we used two-way ANOVA. Statistical differences were considered significant when the *P* value was below 0.05. A single extremely high *synapsin I* ratio (662) in a 3-week-old female rat with markedly low *18S rRNA* quantity was excluded by using the Smirnov test for extreme values ($P < 0.01$). All calculations were performed by using the StatView 5.0 J software for Windows (SAS Inc., Cary, NC, USA).

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