Neonatal sex steroids affect ventilatory responses to aspartic acid and NMDA receptor subunit 1 in rats

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Shi, Yijiang, and Evelyn H. Schlenker. Neonatal sex steroids affect ventilatory responses to aspartic acid and NMDA receptor subunit 1 in rats. J Appl Physiol 92: 2457–2466, 2002.—We hypothesized that administration of estradiol benzoate to males and testosterone propionate to female neonatal rat pups alters sex-specific ventilatory responses to aspartic acid with correspondent changes in N-methyl-D-aspartate receptor subunit 1 (NR1) expression determined by Western blot in specific brain regions. One-day-old rat pups received estradiol benzoate, testosterone propionate, or vehicle and were studied at weaning and adulthood. Different groups had distinct patterns of changes in tidal volume and frequency of breathing after aspartic acid administration. NR1 expression in hypothalamus was altered by age, sex, and treatment. Medullary and pontine NR1 expression correlated with baseline ventilation and magnitude of the ventilatory response to aspartic acid in some groups. Thus 1) tidal volume and breathing frequency patterns in response to aspartic acid are gender, age, and treatment dependent; 2) sex, age, and exogenous steroid hormones affect NR1 expression primarily in the hypothalamus; and 3) there is correlation between NR1 expression in pons and medulla with ventilatory parameters.

estradiol benzoate; testosterone propionate; hypothalamus; pons; medulla; body weight; anogenital distance; gender; N-methyl-D-aspartate

SEXUAL DIMORPHISM IN CONTROL OF BREATHING has been demonstrated by a number of studies (2, 19, 46, 56), including those using aspartic acid, an N-methyl-D-aspartate (NMDA) receptor agonist (48–50). Specifically, 580mg/kg aspartic acid depressed ventilation in male but not in female rats (48). Additionally, dextromethorphan, an NMDA receptor (NR) antagonist, depressed ventilation in female but not in male rats (47). Thus it is plausible that NRs function differently in modulating ventilation in adult female and male rats. Moreover, treatment of neonatal female rat pups with testosterone propionate (TP) resulted in an adult ventilatory response to aspartic acid similar to that of the male adult rats (49) but different from those of intact or ovariectomized female rats. Finally, perinatal estradiol benzoate (EB) treatment of male rat pups resulted in smaller, hypogonadal adult animals whose ventilatory response to hypercapnia was diminished and whose ventilatory responses to aspartic acid were female-like relative to those of control rats (50). All these studies strongly suggest interactions between sex steroid hormones, NRs, growth, and the ventilatory control that may be influenced during critical periods of development.

Native NRs are heterodimers composed of NR subunit 1 (NR1) and NR2A, B, C, and D or NR3A or B subunits (37, 40). The distribution patterns of some of these subunit types differ in male and female rat brains (21) and throughout development (1, 31, 35). Furthermore, there is evidence that sex steroid hormones influence NRs (7, 16, 25, 61, 62). For example, estrogen significantly increases mRNA levels of hypothalamic NR2B in 30-day-old but not in 15-day-old female rats (25). In the anteroventral periventricular nucleus of the hypothalamus, estrogen appears to suppress levels of NR1 mRNA (18). Moreover, systemic estradiol treatment increases the density of NRs (61), NR1 immunofluorescence (16), and postsynaptic sensitivity to NR-mediated synaptic transmission (63) in rat hippocampus.

On the basis of the effects of sex steroids on body growth and development (4, 15, 17, 20, 27), NRs (7, 16, 25, 61, 62) and gender differences in control of ventilation (2, 19, 46, 48–50, 56, 65), we generated several hypotheses. First, neonatal treatment of female rats with TP and male rats with EB will result in a “masculinization” or “feminization,” respectively, of growth and development and in control of ventilation in response to aspartic acid in weanling and adult animals. Second, there is a gender, age, and treatment (TP or EB) difference in protein expression levels of NR1, with Western blotting, in the hypothalamus, pons, and medulla areas that affect growth (32, 34) and also control breathing (43, 59). Third, neonatal treatment of rats with TP and EB will result in changes in NR1 protein expression in these brain regions, which correspond to changes in ventilatory responses to aspartic acid. Finally, increased steroid hormone production during adulthood counteracts the effects of neonatal treatments.
**METHODS**

**Animal Groups and Sex Hormone Treatments**

Sprague-Dawley rat pups were bred at the Lee Medical Animal Facility. After birth, rat pups were sexed according to their anogenital distances (AGD). Neonatal rat pups were treated 1 day after birth with 0.1 ml sesame oil vehicle, 100 μg TP/6 g body wt (suspended in 0.1 ml sesame oil) in females, or 100 μg EB/6 g body wt (suspended in 0.1 ml sesame oil) in males. Animals were housed in plastic cages in a temperature-controlled room (−22°C) on a 12:12-h light-dark cycle with their dams. Food and water were available ad libitum. The University of South Dakota Animal Care and Use Committee approved all procedures.

Rats were weaned 21 days after birth, and the ventilatory response to aspartic acid was measured in half of the rats. Subsequently, rats were weighed, AGDs were measured, and rats were killed and had their brains removed. The remaining rats were raised until adulthood. Procedures described above for weanlings were repeated in the adult rats (~50 days old). There were a total of eight groups of rats with six animals in each group: weanling female TP-treated, weanling female control, weanling male EB-treated, weanling male control, adult female TP-treated, adult female control, adult male EB-treated, and adult male control.

**Ventilation Measurements**

Ventilation was determined in conscious rats placed in a 20 × 8 cm Plexiglas cylindrical chamber. One side of the chamber contained ports to measure 1) the flow rate of air exiting the chamber by using a Gilford flowmeter (Barnant, IL) and 2) the chamber temperature by using a thermometer (Cole Parmer, IL). The other side of the chamber contained ports to 1) allow air to enter the chamber, 2) measure the pressure fluctuations associated with ventilation by use of a low-pressure transducer (Validyne, CA) coupled to a Bio-Pac data acquisition system, and 3) calibrate the system by using a 1-ml glass syringe. Ventilatory parameters determined were tidal volume (VT), frequency of breathing (f), and the end tidal pressure of the bands on the membrane followed by exposure to X-ray development (Pierce) of the polyvinylidene fluoride membrane.

**Protein Assay and Western Blotting**

**Preparation of samples.** Samples of hypothalamus, pons, and medulla from each animal prepared as described above were thawed at 37°C for 5 min and centrifuged at 14,000 g at room temperature for 5 min. Supernatant was removed and diluted 1:3.5 with 67× Laemmlli buffer to make the final concentration of sample solution equal to 1× Laemmlli buffer. Then the solution was mixed, boiled for 5 min, and centrifuged at 14,000 g for 5 s. Finally, the solution was sonicated for 6 s and stored at −70°C.

**Normalization of protein amount.** To evaluate the relative protein concentration of each sample, samples were thawed at 37°C for 5 min, centrifuged at 14,000 g for 5 min, and 10 μl supernatant was taken for loading. Samples were run at 200 V for 20 min, and gels were stained in Coomassie solution [0.0016% Coomassie brilliant blue (Bio-Rad Laboratories), 9.5% ethanol, 5% acetic acid, (66)]. Densitometry was used to determine the integrated density value (IDV) of each lane by using ChemiImager 400 software. According to their IDV values, volumes of samples were adjusted so that amount of protein from each sample was the same in each lane within a gel (14, 39). This volume for each sample was used for the following two procedures.

**Western blotting.** To evaluate the NR1 protein levels, samples were loaded onto the 7.5% polyacrylamide gel and separated by PAGE at 200 V for 30 min. Prestained molecular weight standards (Amersham) were included in each gel to indicate the molecular weight of the protein bands. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride (Immobilon-P, Millipore) membrane in an electroblotting apparatus (Bio-Rad Laboratories) for 1 h at 100 V (transfer buffer: 25 mM Tris base, 192 mM glyoxal, 20% methanol, 0.5% SDS). For immunodetection, blots were blocked for 1 h at room temperature in 2.24 mM Tris base, 7.76 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween 20 (TBST) containing 5% blocker (nonfat dry milk). Incubation with anti-NR1 monoclonal mouse antibody (1:1,000, PharMingen) was carried out for 1 h at 37°C in blocking buffer, followed by 40 min of washing blots with TBST. After washing, blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:1,000, PharMingen) in blocking buffer for 1 h at room temperature, followed by 40 min of washing with TBST. NR1 protein bands were visualized by using the enhanced chemiluminescence development (Pierce) of the polyvinylidene difluoride membrane followed by exposure to X-ray film. Molecular weights of the bands on the film were identified by comparison to the prestained, low-molecular-weight standards that were run in an adjacent well during electrophoresis.

**Verification of relative protein amounts used for the Western blot.** A separate electrophoresis followed by Coomassie staining for each set of samples that was run on the Western blot was used to further assure relatively equal amounts of protein in each lane (Fig. 1). The coefficient of variance (CV; mean divided by standard deviation × 100) for IDVs from this procedure in each gel ranged from 4 to 5%. To evaluate the amount of protein transferred to each blot, 0.2% Naphthal blue black (Sigma Chemical) in the mixture solution of 100% methanol, 100% acetic acid, and H₂O (5:1:5 in volume) was used to stain the blot, followed by washing with the mixture of 100% methanol, 100% acetic acid, and H₂O (1:1:8 in volume). CV for IDV values from this procedure in each blot ranged from 4 to 5%.

**Tissue Collection and Homogenization**

Rats were killed by inhalation of CO₂. To evaluate the effectiveness of the neonatal treatment, AGDs and body weights were measured. To collect the brains, an incision was made in the scalp, and the whole brain was extracted. The hypothalamus, pons, and medulla were then dissected on ice. For 0.1 g of tissue, 300 μl of 2× Laemmlli’s buffer (62.5 mM Tris·HCl (pH 6.8), 25% glyceral, 2% SDS, 5% β-mercaptopro ethanol, and 0.01% bromophenol blue) were added, followed by sonication, using a 550 sonic dismembranator, for 30 s. Disrupted tissues were stored at −70°C.
**RESULTS**

**Effects of Neonatal Treatment with TP or EB on Animal Growth and Development**

**Body weight.** A two-way ANOVA indicated that, in regard to body weight, there was a significant interaction between gender and treatment in adult groups [F(1,23) = 28.26, \( P < 0.001 \)]. In males, neonatal treatment of EB significantly decreased the body weight relative to vehicle-treated males, both at weanling and adulthood (Fig. 2). Neonatal treatment of females with TP significantly increased the body weight at adulthood but not at weanling. Also, adult vehicle-treated males had larger body weights than adult vehicle-treated females. No difference was found between weanling vehicle-treated females and males.

**AGDs.** A two-way ANOVA indicated in regard to AGD that there was significant interaction between gender and treatment in weanling groups [F(1,23) = 41.52, \( P < 0.001 \)] and adult groups [F(1,23) = 17.66, \( P < 0.001 \)]. In males, neonatal treatment with EB significantly decreased AGD relative to that in the vehicle-treated males, both at weanling and adulthood, suggesting a feminization effect of EB (Fig. 3). However, adult female TP-treated rats had a trend for a larger AGD compared with vehicle-treated animals (\( P = 0.06 \)). Therefore, in males, neonatal treatment with 100 \( \mu \)g of EB showed a greater effect on body weight and AGD than neonatal treatment of females with 100 \( \mu \)g of TP. Vehicle-treated males had larger
AGDs than vehicle-treated females, both at weanling and adulthood.

Aspartic Acid-Induced Ventilation

Body weight corrected $\dot{V}E$ at baseline. A three-way ANOVA indicated that there is an interaction between age, sex, and treatment [$F(1,47) = 4.596, P = 0.038$]. Overall there was a very large age effect [$F(1,47) = 44.524, P < 0.001$]. Specifically, adult vehicle-treated females had larger BWCV$\dot{E}$ than adult vehicle-treated males and lower BWCV$\dot{E}$ in adult vehicle-treated males than adult EB-treated males (Fig. 4). There were no significant differences among weanling groups.

Ventilation in response to PBS and aspartic acid. Comparison of the effects of PBS to aspartic acid on VT is depicted in Fig. 5A. In both adult and weanling vehicle- or EB-treated males after aspartic acid treatment, VT was not different relative to the response to PBS. By contrast, in weanling TP-treated and adult vehicle-treated females, aspartic acid induced a significant decrease in VT compared with the effect of PBS. Weanling vehicle-treated and adult TP-treated females did not exhibit a significant change of VT in response to aspartic acid.

The effect of PBS compared with aspartic acid on $f$ is depicted in Fig. 5B. In all females, $f$ decreased after aspartic acid administration, relative to PBS. In weanling vehicle-treated and adult EB-treated males, $f$ decreased after aspartic acid relative to PBS. However, weanling EB-treated and adult vehicle-treated males showed no changes of $f$ in response to aspartic acid.

Differences in $\dot{V}E$ responses to PBS and aspartic acid are depicted in Fig. 5C. All groups of animals showed a significant decrease in $\dot{V}E$ in response to aspartic acid. But the pattern of VT and $f$, resulting in the decrease of $\dot{V}E$, was different among groups (Table 1).

Body weight corrected $\dot{V}E$ change in response to aspartic acid and PBS. A three-way ANOVA indicated that there was a significant effect of gender independent of age and treatment on this parameter [$F(1,47) = 4.908, P = 0.032$]. Adult vehicle-treated females had a larger BWCV$\dot{E}$ (P-A)/P than adult vehicle-treated males, but

Table 1. Summary of changes of VT, $f$, and $\dot{V}E$ induced by acute aspartic acid treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weanling $($VT, f, $\dot{V}E$$)$</td>
<td>Adult $($VT, f, $\dot{V}E$$)$</td>
</tr>
<tr>
<td>Vehicle</td>
<td>NS, S, S</td>
<td>NS, NS, S</td>
</tr>
<tr>
<td>Treated</td>
<td>NS, NS, S</td>
<td>NS, S, S</td>
</tr>
</tbody>
</table>

Summary of tidal volume (VT), breathing frequency ($f$), and minute ventilation ($\dot{V}E$) responses of vehicle-treated and either testosterone propionate (females)- or estradiol benzoate (males)-treated groups of rats at both ages in response to aspartic acid relative to the response to PBS. NS, nonsignificant response; S, significant response to aspartic acid.
adult vehicle-treated males displayed a smaller BWCV \( \dot{E} \) than did adult EB-treated males (Fig. 6). Thus there appear to be gender-specific effects on the ventilation magnitude of the response to aspartic acid.

**Western Blot Analysis of NRI Subunit 1 in the Hypothalamus, Pons, and Medulla**

Samples of hypothalamus, pons, and medulla of all animals were evaluated for their NRI protein levels by using Western blot analysis. One representative blot is shown in Fig. 7. Molecular mass of the NRI protein was 116 kDa, in accordance with other documented findings.

**NRI protein expression in the hypothalamus.** A three-way ANOVA indicated that there were significant interactions between age and gender \( [F(1,47) = 7.651, P = 0.009] \) and between age and treatment \( [F(1,47) = 11.508, P = 0.002] \). In particular, weaning female vehicle-treated rats had significantly higher NRI levels than weaning TP-treated females \( (P = 0.0031) \), than adult vehicle-treated female rats \( (P < 0.0001) \), and than weaning male vehicle-treated rats \( (P = 0.0075) \). (Table 2)

**Correlation Between NRI Levels and Ventilatory Parameters**

In the medulla of adult vehicle-treated females and males, there was a positive correlation between ratios of BWCV \( \dot{E} \) for these groups and the respective ratios of their NRI levels \( (r = 0.826, P < 0.05) \) (Fig. 8A). By contrast, in the medulla, weaning vehicle- and TP-treated females exhibited a trend toward a negative correlation between ratios of NRI expression and their ratios of BWCV \( \dot{E} \) \( (r = -0.804, P = 0.054) \). When the ratios were plotted, the data fit a hyperbola (Fig. 8B).

Further analysis showed the equation of the hyperbola: \( y = 3.34 - 1.77x^2 \) (adjusted \( R^2 = 0.67, P < 0.01 \)), where \( y \) is ratio of BWCV \( \dot{E} \) and \( x \) is NRI ratio. A trend toward a negative correlation was also observed between ratios of NRI levels in pons of weaning vehicle- and EB-treated males and their ratios of BWCV \( \dot{E} \) \( (r = -0.923, P = 0.051) \).

Ratios of NRI protein expression in pons of adult vehicle-treated females and males correlated with their ratios of BWCV \( \dot{E} \) \( (r = 0.851, P < 0.05) \). Moreover, ratios of NRI levels in pons of weaning and adult vehicle-treated males were correlated with ratios of BWCV \( \dot{E} \) \( (r = 0.856, P < 0.05) \) (Fig. 8C).

**DISCUSSION**

**Summary of Major Findings**

The major findings of this study were that 1) neonatal administration of EB in male rats caused a significant decrease of body weight and AGD both at weaning and adulthood compared with vehicle-treated males, whereas neonatal administration of TP in female rats resulted in a significant increase of body weight at adulthood. 2) There were age- and gender-specific effects on BWCV and magnitude of the change of BWCV in response to aspartic acid. 3) There were distinct sex-specific patterns of ventilatory responses (VT and \( \dot{f} \)) to aspartic acid. 4) NRI protein expression varied in different brain regions among the eight groups. In the hypothalamus, weaning vehicle-treated females had significantly higher NRI levels than weaning TP-treated females, weaning vehicle-treated males, and adult vehicle-treated females. These differences were not noted in either the pons or the medulla. 5) There was a correlation between ratios of NRI levels of animals with their ratios of BWCV \( \dot{E} \) and the BWCV \( \dot{E} \) \( (P-A)/P \) ratios. Ratios of the NRI levels in medulla of adult vehicle-treated females and males were positively correlated with their ratios of BWCV \( \dot{E} \). Also, ratios of NRI levels in pons between adult vehicle-treated females and males and between weaning and adult vehicle-treated males had a positive correlation.

Table 2. Summary of IDV ratios of NRI1 in hypothalamus of different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>WFV</th>
<th>WFT</th>
<th>WMV</th>
<th>WMT</th>
<th>AFV</th>
<th>AFT</th>
<th>AMV</th>
<th>AMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>0.72</td>
<td>0.63</td>
<td>0.59</td>
<td>0.42</td>
<td>0.83</td>
<td>0.83</td>
<td>1.13</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>0.87</td>
<td>0.86</td>
<td>0.67</td>
<td>0.37</td>
<td>0.33</td>
<td>1.12</td>
<td>0.77</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>0.42</td>
<td>0.39</td>
<td>0.53</td>
<td>0.45</td>
<td>0.74</td>
<td>0.50</td>
<td>0.37</td>
</tr>
<tr>
<td>4</td>
<td>1.00</td>
<td>0.45</td>
<td>0.71</td>
<td>0.55</td>
<td>0.30</td>
<td>0.33</td>
<td>0.37</td>
<td>0.23</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>0.55</td>
<td>0.76</td>
<td>0.31</td>
<td>0.43</td>
<td>0.68</td>
<td>0.30</td>
<td>1.13</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>0.33</td>
<td>0.83</td>
<td>0.61</td>
<td>0.39</td>
<td>0.41</td>
<td>0.57</td>
<td>0.59</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.56 ± 0.20</td>
<td>0.70 ± 0.17</td>
<td>0.54 ± 0.12</td>
<td>0.39 ± 0.05</td>
<td>0.55 ± 0.22</td>
<td>0.62 ± 0.31</td>
<td>0.70 ± 0.38</td>
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</tr>
</tbody>
</table>

For each blot, integrated density values (IDVs) of weaning vehicle-treated females were set as 1 in each Western blot, and IDVs of samples from other animals were expressed as ratios relative to 1. *Level of significance when comparing that group with weaning vehicle-treated females. WFV, weaning female vehicle; WFT, weaning female treated; WMV, weaning male vehicle; WMT, weaning male treated; AFV, adult female vehicle; AFT, adult female treated; AMV, adult male vehicle; AMT, adult male treated; NR1, N-methyl-D-aspartate receptor subunit 1.
with the BWC\textsubscript{V} (P-A)/P ratios. The following sections will address each of these issues in detail.

**Effects of Neonatal Treatment of EB in Males and TP in Females on Growth and Development**

Body weight, an important physiological parameter that shows sexual dimorphism (males being heavier than females), depends on energy intake, expenditure, and storage. Regulation of these processes requires a complex network involving diverse neurochemical and neuroendocrine signals from different organs in the body and integration within several regions in the brain (32, 34). In particular, the hypothalamus plays an important integrative function in this process by acting through a variety of systems that involve an interaction between nutrients, amines (noradrenaline, dopamine, serotonin), neuropeptides (neuropeptide Y, peptide YY, galanin, opioids, growth hormone-releasing factor), and hormones (thyroid hormones, estrogen, testosterone, growth hormone) (32).

Sex steroid hormones may regulate body weight through their interactions with these hormones, neurotransmitters, and neuromodulators, affecting food intake and energy utilization. Perinatal administration of sex steroid hormones resulted in an enduring effect on body weight. For example, Bell and Zucker (3) reported that female rat pups treated with TP (1 mg) or EB (50 \(\mu\)g) on day 5 weighed more than oil-treated controls at adulthood. One-day-old male rats treated with EB (100 \(\mu\)g) weighed less and were shorter as adult rats than controls (50).

In our study, neonatal EB treatment resulted in a decreased body weight both at weanling and adulthood, which reflected the weight-limiting effect of estrogen. As for neonatal treatment of TP in females, the weight-promoting effect of testosterone was not observed until adulthood. Tarttelin et al. (55) studied the effects of different doses of TP and showed that when 90 \(\mu\)g was given on postnatal day 3, the female TP-treated rats began to show greater weight gain than controls from week 5 until the end of experiment (week 18). In another study (10), neonatal testosterone (100 \(\mu\)g) treatment of female rats led to an increase in body weight over a period from day 38 to 76 after treatment. As in the present study, animals were weaned at week 3 in both studies. Therefore, the finding that neonatal treatment of females with TP increased body weight in adulthood (~50 days of age) but not in weanling (~21 days of age) is consistent with previous reports.

Another developmental parameter, AGD, has been demonstrated to correlate with size of sexually dimorphic nucleus-preoptic area (SDN-POA) and sexual behavior. For example, administration of testosterone or estrogen during the critical period (late fetal and early neonatal life) increased the size of SDN-POA and AGD in females (17, 54). One day-old female rats with longer AGDs (>1.4 mm) had significantly larger SDN-POA volumes than 1-day-old female rats with short AGD (≤1.4 mm) as adults (13). Finally androgenized females had smaller SDN-POA than their control females (38).

In this study, AGD was smaller in EB-treated male rats compared with controls, both at weanling and adulthood, and reflected the feminization effect of estrogen. In contrast, TP-treated females did not exhibit an AGD that was significantly different from vehicle controls at either age, but adult treated female rats showed a borderline larger AGD (\(P = 0.06\)). This lack of change in AGD could be related to the dose of TP or a small sample size. In Juarez et al.’s (23) study, pregnant rats were injected with 2 mg TP, and female offspring had larger AGDs at 10, 30, 45, 60, and 75

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**Fig. 8.** A: correlation between ratios of N-methyl-D-aspartate receptor subunit 1 (NR1) levels in the medulla of AFV and AMV and their respective ratios of BWC\textsubscript{V}. B: correlation between ratios of NR1 levels in the medulla of WFV and WFT and their respective ratios of BWC\textsubscript{V}. Here, the data fit into an exponential equation of \(y = 3.34 - 1.77x^2\) (adjusted \(R^2 = 0.67, P < 0.01\)), where \(y\) is the ratio of BWC\textsubscript{V} and \(x\) is the NR1 ratio. C: correlation between ratios of NR1 levels in the pons of WMV and AMV and AFV and AMV and their respective ratios of change of BWC\textsubscript{V} (P-A)/P.
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days of age. Therefore, in our study, TP at a dose of 100 μg appeared not to exert a masculine effect on AGD, although it did show such effect on body weight.

Effects of Neonatal Treatment on Ventilation, Including Aspartic Acid-Induced Ventilation

Differences in BWCV among various groups may reflect effects of neonatal treatment, age, and sex on ventilation. Effects of age were apparent in both female and male rats, with weanlings exhibiting a higher BWCV than adults. This may be related to the higher metabolism rate of weanlings compared with adults (Inamdar and Schlenker, unpublished results). Moreover, there was a sex difference in this parameter in adults but not in weanlings. Gender differences also existed in BWCV (P-A)/P in adult vehicle-treated rats but not in weanlings. Thus puberty could play a role in modulating ventilatory responses to aspartic acid (22).

In previous studies, aspartic acid has been shown to affect ventilation differently in male and female Sprague-Dawley rats and was influenced by neonatal administration of exogenous sex steroid hormones (48). A dose of 580 mg/kg aspartic acid depressed ventilation in male rats for 45 min by decreasing VT, inspiratory flow rate, and f. In contrast, female rats exhibited a transient decrease of ventilation only at 15 min (48). Adult female rats androgenized by the administration of 1 mg TP 1 day after birth responded to aspartic acid administration with a marked depression of ventilation similar to that noted above in males (49). Moreover, aspartic acid administration depressed ventilation in intact adult males but had no effect on ventilation in adult male rats that had received 100 μg of EB 5 days after birth (50).

Subcutaneous injection of aspartate increases concentrations of aspartate within 15 min in the circumventricular organ regions of the brain, including the arcuate nucleus of the hypothalamus, organum vasculosum of the lamina terminalis, and subfornical (44, 45). In contrast, no appreciable increases were detected in other brain regions, such as ventromedial hypothalamic nuclei and medial preoptic nucleus (44, 45). Thus systemic administration of aspartic acid can act on specific areas of the hypothalamus. Moreover, systemically administered aspartic acid may affect the release of prolactin, β-endorphins, and luteinizing and growth hormone-releasing hormones (5, 30, 57). In particular, subcutaneous administration of aspartic acid can affect ventilation by releasing endogenous opioids and somatostatin (51). Thus aspartic acid may act directly (on NRs) and indirectly (by affecting the release of neuromodulators) to affect breathing.

In the present study, all eight groups decreased Ve after aspartic acid administration relative to PBS. However, each group showed a distinct pattern of VT and f response, suggesting sexual dimorphism of control of ventilation in response to aspartic acid (Table 1). In response to aspartic acid, all males showed no change in VT, whereas all females showed a significant change in f. These two ventilatory characteristics may be considered as genetically determined by sex but not affected by neonatal treatment. We call them “male-pattern” and “female-pattern,” respectively. Female and male weanling vehicle-treated rats exhibited similar changes in VT, f, and Ve in response to aspartic acid, suggesting that during the weanling period this pattern of response to aspartic acid may result from low levels of circulating sex steroid hormones.

In response to aspartic acid, adult vehicle-treated males showed no change in f, a pattern that was also found in weanling EB-treated males, suggesting that neonatal treatment with EB induces a response similar to that noted when male animals are mature. This may indicate that, in weanling males, treatment with EB has a “masculine” effect. In contrast, when comparing adult vehicle and adult EB-treated males, the latter showed a decrease in f, which is the female-pattern change, suggesting that, after puberty, EB treatment in males will induce a feminization effect.

When comparing weanling and adult vehicle-treated females, the latter showed a significant decrease in VT, which was also found in weanling TP-treated females, suggesting that neonatal treatment of TP in females induces a change that normally happens when animals are mature. Comparing adult vehicle and adult TP-treated females, the latter showed no change in VT in response to aspartic acid, which is the male-pattern change. This may suggest that, after puberty, TP treatment in females will induce a masculine effect.

The discrepancy in Ve response to aspartic acid between this study and earlier reports (48–50) could potentially relate to several factors, such as different doses of EB or TP used in the studies, times when EB and TP were given, individual genetic variability within each group, and different commercial sources of Sprague-Dawley rats. In the studies by Schlenker and colleagues (48–50), rats were obtained from Sasco (Omaha, NE), whereas this study used rats obtained from Harlan (Madison, WI). Moreover, rats obtained from Hilltop (Scottsdale, PA) also showed a different sex-specific ventilatory pattern of response to aspartic acid (22). In that study, female vehicle-treated rats decreased Ve after aspartic acid administration both at weaning and adulthood, whereas weanling but not adult male vehicle-treated rats decreased Ve after aspartic acid administration. Furthermore, female rats treated with 50 μg TP neonatally showed no change in Ve after aspartic acid administration both at weaning and adulthood (22). That different rat strains demonstrate variability in sexual differentiation in behavior and brain structure (33) suggests the importance of genetic factors and sexual differentiation. Thus the ventilatory response of rats to aspartic acid appears to be modulated by genetic factors beyond sex influence.

NR1 Protein Levels in Hypothalamus, Pons, and Medulla

We examined the relative NR1 protein expression in three brain regions of each group to see whether
changes in NR1 levels were associated with ventilatory responses to aspartic acid in male and female rats of two age groups who had received different neonatal treatments. The areas of hypothalamus, pons, and medulla were selected because of their important roles in the control of ventilation (43, 59) and also the sexual dimorphism noted in hypothalamic nuclei (36, 52, 58). The role of pons and medulla has been well described in pattern generation (43), and the hypothalamus can modulate ventilatory patterns (59). Moreover, hypothalamic nuclei such as the paraventricular nucleus, the arcuate nucleus, and caudal nuclei send projections to brain areas associated with ventilatory control, such as nucleus of the tractus solitarius, nucleus ambiguus, phrenic motoneurons, and parabrachial nuclei (28, 64, 67). Moreover these brain regions contain NR (12, 41). Ventral periventricular nucleus, and ventromedial hypothalamic nuclei show a high percentage of NR1 expression than neonates within the nucleus of the tractus solitarius, nucleus ambiguous, phrenic motoneurons, and parabrachial nuclei (28, 64, 67). Moreover these brain regions contain NR (12, 41).

NR subunits also exhibit temporal and developmental patterns of distribution in the central nervous system (1, 31, 35). For example, in five male rat brain regions (olfactory bulb, cortex, hippocampus, midbrain, and cerebellum), levels of NR1 protein were low at birth and increased in similar patterns of 2- to 4.5-fold from P2 to P42 (35). Moreover, relationships between NR subunits and sex steroid hormones exist (16, 25, 61, 63). In the present study, there was an effect of age on NR1 levels in hypothalamus. In particular, weanlings had higher NR1 levels within the hypothalamus than adults, dependent on sex and treatment. A similar trend was noted in the medulla.

Most studies investigating developmental effects on NR1 levels have not done so in the hypothalamus, pons, or medulla in weanlings and adults. In contrast, Ohtake et al. (42) showed that adult rats had higher NR1 expression than neonates within the nucleus of the tractus solitarius, with the situation being reversed in the hypoglossal nucleus. In the present study, we noted a decrease of NR1 expression in the hypothalamus and a similar trend in the medulla after sexual maturity.

The hypothalamus is likely a target site for sex steroid hormone effects in the central nervous system. This includes the SDN-POA, arcuate nucleus, anteroventral periventricular nucleus, and ventromedial hypothalamic nucleus (52). Estrogen receptors (8, 9, 24) and NR1 mRNA are contained in many nuclei in the hypothalamus (12). Moreover, the ventromedial nucleus of the hypothalamus shows a high percentage of colocalization of estrogen receptor α and NR2D mRNA (26). Thus there could be interaction between estrogen, estrogen receptors, and NR subunits in specific hypothalamic nuclei. The present study is the first study to compare NR1 protein levels in the hypothalamus according to age, sex, and neonatal treatment. In the present study, weanling vehicle-treated females had higher NR1 levels than weanling TP-treated females. Although we did not measure estrogen receptor levels, it is probable that weanling vehicle-treated rats had higher estrogen receptor levels than weanling female TP-treated rats because treatment of female rats with TP significantly decreases the E2-receptor content in the pituitary and hypothalamus (29, 53). Thus there could be a correlation between estrogen receptor levels and NR1 levels in the hypothalamus that are affected by neonatal perturbations.

Because we did not examine NR1 levels in specific hypothalamic nuclei, our results represent the overall protein expression of NR1 within the entire hypothalamus. If sex steroid hormones in different hypothalamic nuclei regulate NR1 levels differently, the Western blotting technique as applied in this study is not sensitive enough to investigate regional effects of sex steroid hormones. To overcome this shortcoming, immunohistochemical studies could be used to investigate NR1 expression in specific hypothalamic neurons in rats of different ages, sex, and neonatal treatments.

Correlation Between BWCved, BWCvex (P-A)/P, and NR1 Levels in Pons and Medulla

The ratio of NR1 levels of adult vehicle-treated females to males in the pons and medulla, areas associated with control of breathing, correlated positively with BWCved (P-A)/P and BWCvex, respectively. Moreover, there was a positive correlation between the ratio of NR1 levels in pons of weanling and adult vehicle-treated males and the ratio of their BWCved (P-A)/P. However, ratios of NR1 levels in medulla of weanling vehicle- and TP-treated females and ratios of their BWCved exhibited a hyperbolic relationship ($y = 3.34 - 1.77x^2$, adjusted $R^2 = 0.67$, $P < 0.01$). Thus the role of NRs in the pons and medulla on control of ventilation may be influenced by sex, treatment, and age.

In some groups, however, we found no correlation between NR1 levels and the ventilatory response to aspartic acid. This could result from several factors. First, distinct patterns of ventilatory response to aspartic acid could result from effects of sex steroids on subunit expression, channel function, such as altering phosphorylation of NR (7, 11, 18, 25, 62), rather than only a change of NR levels. For example, the NR2D gene, which contains estrogen response elements (60), could be the target of sex steroids and may contribute to the distinctive sex-specific ventilatory responses to aspartic acid. Second, subunit composition could change without any identifiable change in the total number of NRs (6), as evaluated by NR1 levels. To further explore the relationship between NRs, sex steroid hormones and their roles in control of ventilation, evaluation of other NR2 subunits and estrogen receptor-α and -β protein levels in brain regions associated with control of breathing is needed.

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