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Author(s)	Wada, Hiromi; Yumoto, Shoko; Iso, Hiroyuki
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Title Page

Title

IRREVERSIBLE DAMAGES TO AUDITORY SYSTEM FUNCTIONS
CAUSED BY PERINATAL HYPOTHYROIDISM IN RATS

Author Names and Affiliations

Hiromi Wada ^a, Shoko Yumoto ^a, Hiroyuki Iso ^b

^a Graduate School of Letters, Hokkaido University, Kita 10 Nishi 7 Kita-Ku,
Sapporo 060-0810, JAPAN

wada@let.hokudai.ac.jp

^b Division of Education Center, Hyogo University of Health Sciences,
Minatojima 1-3-6, Chuo-Ku, Kobe 650-6530, JAPAN

Corresponding Author

Name: Hiromi Wada

Address: Graduate School of Letters, Hokkaido University, Kita 10 Nishi 7
Kita-Ku, Sapporo 060-0810, JAPAN

Tel: +81-11-706-3321, Fax: +81-11-706-3321

E-mail: wada@let.hokudai.ac.jp

Abstract

We examined the effect of perinatal hypothyroidism on auditory function in rats using a prepulse inhibition paradigm. Pregnant rats were treated with the antithyroid drug methimazole (1-methyl-2-mercaptoimidazole) from gestational day 15 to postnatal day 21 via drinking water at concentrations (w/v) of 0 (control), 0.002 (low dose), or 0.02% (high dose). Rats from methimazole-treated mothers were tested at ages 1, 6, and 12 months using techniques to examine prepulse inhibition and startle response. The startle stimulus consisted of 40 ms of white noise at 115 dB, whereas the prepulse, which preceded the startle stimulus by 30 ms, consisted of 20 ms of white noise at 75, 85, or 95 dB. When the prepulse intensity was 75 or 85 dB, the high-dose group showed decreased prepulse inhibition percentages compared with the control and low-dose groups. The reduced percentages of prepulse inhibition did not return to control levels over the 12-month study period. In contrast, no differences in prepulse inhibition were observed among the three dose groups when prepulse intensity was 95 dB. Moreover, the high-dose group displayed excessive reaction to auditory startle stimuli compared with the other groups. Reductions in plasma free thyroxine and body weight gain were observed in the high-dose group. We conclude that perinatal hypothyroidism results in irreversible damage to auditory function in rats.

Key Words: auditory function; hearing loss; hypothyroidism; methimazole; prepulse

inhibition; rat; startle response; thyroxin

1. Introduction

Thyroid hormones are essential for normal brain development because they regulate neuronal proliferation, migration, and differentiation (Porterfield, 1994, 2000). In pregnant women, thyroid hormone deficiencies result in cretinism in the offspring (Boyages and Halpern, 1993). The neurological features of cretinism include speech disturbances, mental retardation, gait disorders, and deafness. Patients with cretinism commonly display auditory deficits (Boyages and Halpern, 1993), suggesting that the auditory system is vulnerable to thyroid hormone deficiency (Goldey et al., 1995).

Auditory function can be tested with prepulse inhibition (PPI) or reflex modification. PPI involves inhibiting the startle response to an auditory stimulus with a high intensity pulse (P) when P is preceded by a non-startling, low-intensity stimulus prepulse (PP). The general procedure fixes the intensity of P and systematically varies the intensity of PP. The minimum intensity of PP that induces PPI is the auditory threshold for the effect of PP on the startle response (Crofton, 1990). PPI offers many benefits. First, PPI is applicable to early developmental stages in rats because the auditory startle response develops by postnatal day (PND) 12 (Schneider and Golden, 1986, 1987). Second, PPI does not require any preliminary training to acquire the startle response. Third, PPI can be achieved in mice, rats, rabbits, pigeons, and human

infants and adults (Hoffman and Ison, 1980). Because the neurobiological processes of PPI are similar in mammalian species (Crofton, 1990), it may be possible to extrapolate the results from animal experiments to humans.

The relationships between thyroid hormone deficiency and auditory deficits have been demonstrated in animal models using PPI of the auditory startle response (Goldey et al., 1995; Henck et al., 1996; Schneider and Golden, 1986, 1987). Pregnant rats treated with antithyroid drugs such as methimazole (MMI) or propylthiouracil (PTU) give birth to pups with perinatal hypothyroidism. The auditory threshold of PP required to induce PPI is elevated in rats exposed to PTU (Goldey et al., 1995). In addition, MMI-treated rats have a delay in the acquisition of the startle response in a dose-dependent manner from PND 15.2 to 17.8 compared with control rats on PND 12 (Schneider and Golden, 1986). Perinatal hypothyroidism decreases the amplitude of the startle response on PND 17–24, whereas it enlarges the amplitude on PND 43 or 75 (Goldey et al., 1995; Henck et al., 1996).

However, the long-term effect of perinatal hypothyroidism on auditory function remains unresolved. PTU-treated rats show elevated auditory brainstem response (ABR) thresholds that do not recover 8–9 weeks after birth (Axelstad et al., 2008). MMI treatment also causes an ABR waveform with a slower latency and altered shape compared with controls even on PND 90 (Albee et al., 1989). Further studies are necessary to clarify whether perinatal hypothyroidism irreversibly damages auditory

function.

In this study, we examined the long-term effect of perinatal hypothyroidism on auditory function using the PPI paradigm. Pregnant rats were treated with MMI and the pups were tested over a 12-month period after birth. We predicted that the auditory threshold required to induce PPI would be elevated and the amplitude of the startle response would be higher over the 12-month period.

2. Materials and Methods

2.1. Subjects

Twenty-four pregnant Wistar rats were purchased at gestational day (GD) 8 from Japan SLC Inc. (Hamamatsu, Japan). The animals were housed in individual cages, supplied with the certified rat chow MF (Oriental Yeast Ltd., Sapporo, Japan) *ad libitum*, and randomly assigned to either a control group (n = 8), a low-dose group (n = 8), or a high-dose group (n = 8). The antithyroid drug MMI (Sigma Aldrich Co., MO, USA) was dissolved in distilled water and administered to the animals via drinking water. MMI at concentrations (w/v) of 0 (control), 0.002 (low dose), or 0.02% (high dose) was administered to the rats starting on GD 15 until PND 21. MMI treatments of 0.001–0.005% are the lowest doses that cause hypothyroidism in adult rats; hence, 0.002% MMI treatment was considered as the low dose in this study (Cooper, et al., 1984). Moreover, treatment with 0.02% MMI from GD 15 to PND 21 affects

myelinogenesis in rat brain (Barradas, et al., 2000). Two pups (one male and one female) were sampled from each litter after weaning on PND 21. Pups in the high-dose group were weaned on PND 28 because of developmental delay. The mothers of these pups were given tap water from PND 22 to 28. Eight pups were assigned to each of the following groups: male control (MC), male low-dose (ML), male high-dose (MH), female control (FC), female low-dose (FL), or female high-dose (FH) groups. Two or three pups from each group were housed per cage. The certified rat chow MF and tap water were provided to the pups *ad libitum*.

Room temperature was maintained at $22 \pm 2^\circ\text{C}$ with a relative humidity of $50 \pm 10\%$. The rat pups were kept under a 12-h light/dark cycle (light, 19:00–07:00 h; dark, 07:00–19:00 h), and PPI and startle responses were analyzed during the dark period. This protocol was approved by the Center for Advanced Science and Technology (Hokkaido University). All conditions complied with the Guide for the Care and Use of Laboratory Animals of Hokkaido University.

2.2. Apparatus

The experimental chamber was a clear acrylic cage ($15 \times 22 \times 12$ cm) with aluminum mesh walls on two sides. A piezoelectric accelerometer (GH313A, GA-245SO; KEYENCE, Osaka, Japan) attached underneath the experimental chamber detected movements of the rats and transduced them into voltage outputs. The voltage outputs were digitized at 1000 Hz and recorded on a personal computer through a 60

Hz low-pass filter. White noise was used as P and PP. The white noise was amplified by a speaker placed adjacent to the experimental chamber. Both the experimental chamber and the speaker were placed within a sound-insulated box to attenuate external sound and light. Background noise was maintained at a constant level of 70 dB throughout testing.

2.3. Auditory startle response and PPI

Rats were habituated to the experimental chamber in the presence of 70 dB continuous background noise for 5 min and then underwent both startle response and PPI testing. The rats were exposed to 115 dB of P for 40 ms for startle response testing. A startle response was defined as the difference between the maximum and minimum peak amplitudes of the voltage outputs within a 200-ms period after the onset of P. Startle response testing consisted of 10 trials per day for 3 days. The inter-trial intervals (ITIs) were varied, and the mean ITI was 20 s.

Rats were exposed to either P alone (P trial) or P with a preceding PP (PP trial) for PPI testing. A 115-dB 40-ms P was presented in P trials. PP with an intensity of 75, 85, or 95 dB for 20 ms was presented 30 ms before P in PP trials. The intensity of PP was changed daily for 3 days. Half of the rats received PPs with ascending intensities of 75, 85, and 95 dB, whereas the other half received PPs with descending intensities of 95, 85, and 75 dB. PPI testing consisted of a pseudo-random presentation of eight P trials and 10 PP trials per day for 3 days. The percentage PPI was calculated

by the following formula: % PPI = $[(P - PP)/P] \times 100$, where P and PP are averages of the startle response amplitudes of P and PP trials, respectively. The ITIs were varied, and the mean ITI was 20 s.

The rats were tested for PPI and startle responses for 3 days at 1, 6, and 12 months after birth. In other words, the rats were repeatedly tested at these three time points.

2.4. Determination of thyroid hormones

Free triiodothyronine (FT3), free thyroxine (FT4), and thyroid-stimulating hormone (TSH) were determined from the same set of MMI-treated animals. Whole blood was collected from the abdominal aorta of ether-anesthetized pups on PND 21, centrifuged at 3000 rpm for 10 min, and the plasma was stored in a microtube. The FT3 and FT4 concentrations were determined using the ACS-FT3 II and LKFT41 kits, respectively (Siemens Healthcare Diagnostics Co., Tokyo, Japan). TSH concentrations were determined using the rat TSH ELISA kit (R-type) (Shibayagi Co., Shibukawa, Japan). All assays were performed at Mitsubishi Chemical Medience (Tokyo, Japan).

2.5. Statistical analysis

The percentages of PPI were analyzed by a four-factor analysis of variance (ANOVA) between subject variables of dose and sex and within subject variables of PP intensity and age. Startle response amplitudes and body weights were analyzed by a three-factor ANOVA between subject variables of dose and sex and within subject

variables of age. A two-factor ANOVA was used between subject variables of dose and sex to analyze plasma FT3, FT4, and TSH concentrations as well as body weights on PND 21. Ryan's method was used for multiple comparison tests when a primary effect was significant. These statistical analyses were executed using ANOVA 4 on the Web (<http://www.hju.ac.jp/~kiriki/anova4/about.html>).

3. Results

3.1 Percentage of PPI

The effects of dose, PP intensity, and age were significant [$F(2,42) = 16.385$, $p < 0.001$; $F(2,84) = 52.342$, $p < 0.001$; $F(2,84) = 24.608$, $p < 0.001$]. An interaction was observed between dose and PP intensity [$F(4,84) = 9.991$, $p < 0.001$]. When the PP intensity was 75 dB or 85 dB, the high-dose group showed decreased percentage PPI compared with both the control and low-dose groups ($ps < 0.05$) (Fig. 1a). However, no significant difference was observed among the three dose groups when a PP of 95 dB intensity was presented. The interaction between dose and age was also significant [$F(4, 84) = 8.834$, $p < 0.001$]. No significant difference in percentage PPI was observed among the three dosage groups at 1 month, but the high-dose group displayed lower percentage PPI than both the control and low-dose groups at 6 and 12 months ($ps < 0.05$) (Fig. 1b). The control and low-dose groups showed an increased percentage PPI at 6 and 12 months compared with that at 1 month ($ps < 0.05$), whereas the high-dose

group did not show an increased percentage PPI over the 12-month period. No significant difference in percentage PPI was observed between the control and low-dose groups. Sex difference did not affect the percentage PPI, and none of the three-factor or four-factor interactions was significant.

[Insert Figure 1]

3.2 Startle response

Dosage and sex significantly affected the startle response [$F(2,42) = 30.347$, $p < 0.001$; $F(1,42) = 34.519$, $p < 0.001$], and the interaction between dose and sex was significant [$F(24,128) = 3.918$, $p < 0.001$]. The MH group exhibited greater amplitude than both the MC and ML groups ($ps < 0.05$) (Fig. 2). The FH group also exhibited higher amplitude than both the FC and FL groups ($ps < 0.05$). Age significantly affected the startle response [$F(2,84) = 28.930$, $p < 0.001$]. Startle response amplitude was lowest at 1 month compared to that at ages 6 and 12 months ($ps < 0.05$). Startle response amplitude was not significantly different between the control and low-dose groups. None of the other interactions was significant. A sex difference was observed in startle response amplitude. The male group showed an increased amplitude of startle responses compared with that in the female group. Lehmann et al. (1999) reported that body weight differences in males and females affect startle response amplitudes. Hence, we analyzed the covariance with body weights for startle response amplitudes using the pooled data at 1, 6, and 12 months of age. Significant differences were still observed

[F(1,141) = 12.460, $p < 0.001$], indicating that other factors affected startle response amplitudes.

[Insert Fig. 2]

3.3 Body weight

Significant effects of dose, sex, and age on body weight gain were observed [F(2,42) = 21.967, $p < 0.001$; F(1,42) = 1166.340, $p < 0.001$; F(2,84) = 4183.921, $p < 0.001$]. An interaction was identified between dose and sex [F(2,42) = 3.385, $p < 0.05$]. The MH group gained significantly less body weight than both the MC and ML groups ($ps < 0.05$) (Fig.3a). The FH group also displayed a lower body weight than both the FC and FL groups ($ps < 0.05$). The male groups gained more body weight than that in the female groups over the 12-month period ($ps < 0.05$). In addition, the interaction between dose and age was significant [F(4,84) = 8.532, $p < 0.001$]. The high-dose group gained less body weight compared with both the control and low-dose groups at 1 and 12 months of age ($ps < 0.05$) (Fig. 3b). No significant difference in body weight gain was observed between the control and low-dose groups. None of the three-factor interactions was significant.

[Insert Fig. 3]

3.4 Thyroid hormone concentrations

Dosage significantly affected FT4 concentration on PND 21 [F(2,61) = 27.142, $p < 0.001$]. The high-dose group showed a decreased FT4 concentration

compared with that in both the control and low-dose groups ($p < 0.05$) (Fig. 4a). No effect of dosage on either FT3 or TSH concentrations was observed (Fig. 4b and c). Sex difference did not affect FT3, FT4, or TSH concentrations. The body weight gain on PND 21 was affected by dosage [$F(2,62) = 59.941, p < 0.001$]. The high-dose group gained less body weights than that the control and low-dose groups ($p < 0.05$) (Fig. 4d). The effect of sex difference was significant for body weight gain [$F(1,62) = 4.967, p < 0.05$]. The male group gained more body weights than that the female group. No significant differences were observed between the control and low-dose groups for FT4, FT3, and TSH concentrations or body weight gain. No interaction between dose and sex was significant for FT3, FT4, and TSH concentrations, or body weight gain.

[Insert Fig. 4]

4. Discussion

Perinatal hypothyroidism results in auditory dysfunction in animals. Pups produced by pregnant rats treated with antithyroid drugs have elevated auditory thresholds required to induce PPI and ABR (Axelstad et al., 2008; Goldey et al., 1995; Knipper, et al., 2000). Hypothyroid pups have retarded acquisition of the startle response to auditory stimuli, but amplitude increases subsequently (Goldey et al., 1995; Henck et al., 1996; Schneider and Golden, 1986, 1987). The long-term effect of perinatal hypothyroidism on auditory function remains unresolved. Only a few studies

have examined auditory function in adulthood (Albee et al., 1989; Axelstad et al., 2008; Goldey et al., 1995; Knipper, et al., 2000). We treated pregnant rats with MMI at concentrations of 0.002% and 0.02% and tested the auditory function of the pups using PPI and startle response techniques over a 12-month period.

We found that the high-dose group had reduced percentage PPI compared with both the control and low-dose groups; the PP intensity was 75 or 85 dB. Rats in all groups exhibited the same percentage PPI levels when the PP intensity was 95 dB. The high-dose group required more intense PP to yield a similar percentage PPI compared with that in the other groups. These findings are consistent with previous studies indicating that hypothyroid animals have increased auditory thresholds required to cause PPI (Crofton et al., 2000a; Crofton et al., 2000b; Goldey et al., 1995), suggesting the possibility of low intensity hearing loss. Perinatal hypothyroidism damages the cochlear structure. For example, the tectorial membrane of the cochlear canal becomes distorted and does not connect with the hair cells (Uziel et al., 1981). Many of the outer hair cells are lost, and both afferent and efferent innervation to the hair cells degenerates (Uziel et al., 1983). The decrease in outer hair cell activity is not localized to specific areas but spans whole frequency areas of the cochlear basilar membrane (Axelstad et al., 2008). Unfortunately, these structural abnormalities are irreversible even after the thyroid deficiency is relieved (Uziel et al., 1983). It is possible that the low-intensity hearing loss in our high-dose group may have been a result of structural

abnormalities of the cochlea caused by thyroid deficiency.

In our study, MMI reduced the percentage PPI in 6- and 12-month-old pups but did not affect PPI in 1-month-old pups. Body weight gains in 1-month-old pups were considerably lower compared with those in 6- and 12-month-old pups (Fig. 3). The piezoelectric accelerometer might be less sensitive to detect the startle response of low-weight animals.

PPI paradigms usually elevate PP intensities from 72 dB to 85 dB with 2 to 4 dB steps, and stepwise increases are observed (Lehmann et al., 1999; Van den Buuse and Eikelis, 2001). Our study showed a significant increase in the percentage PPI for PP intensities between 75 dB and 85 dB but no difference was observed between 85 dB and 95 dB PPs. Both 85 dB and 95 dB PPs caused maximum inhibition of startle responses and leave no further room for a stepwise increase. Further studies are necessary to obtain a stepwise function by testing PP levels that are lower than 85 dB. PP intensities greater than 85 dB may reduce the sensitivity of percentage PPI assay because of the lack of a stepwise curve.

Acquisition of the auditory startle response is delayed in perinatal hypothyroid rats (Henck et al., 1996; Schneider and Golden, 1986, 1987), which may be associated with structural abnormalities of the cochlea. In contrast, the neural mechanisms that underlie increased startle responses are unknown. One possibility is that inner hair cells compensate for the function of outer hair cells. Although

degeneration of the outer hair cells is irreversible, the inner hair cells and their afferent and efferent innervations are normal. Hearing onset occurs a few days after the stopping PTU treatments (Uziel et al., 1983). The increased amplitude of the startle responses observed may occur due to excessive functioning of inner hair cells. Another possibility is that hypothyroidism affected the formation of auditory startle response neural circuits, which are composed of cochlear root neurons, neurons in the nucleus reticularis pontis caudalis, and spinal motoneurons (Lee, et al., 1996). Thyroid hormones regulate the expression of genes involved in axonal growth, myelination, and synaptic formation (Barradas, et al., 2000; Knipper et al., 1998; Kobayashi et al., 2005). Malformation of auditory startle circuits due to abnormal neural connections may underlie excessive startle responses. Further studies are necessary to elucidate the neural mechanisms of the startle response in hypothyroid animals. In addition, a sex difference was observed in the amplitudes of the auditory startle responses; the amplitude in the male group was greater than that in the female group. This result is comparable with previous reports (Goldey et al., 1995; Lehmann et al., 1999). A study by Lehmann et al. (1999) that used a piezoelectric accelerometer to measure startle response amplitudes suggested that body weight differences in males and females result in startle response differences. However, in our study, the difference in startle response amplitudes persisted even after adjusting for body weight differences. Other factors, such as greater muscle mass in males, might be responsible for the increased

startle responses (Ison and Allen, 2007).

We determined FT4 and FT3 levels because these free hormones are more active than the binding ones and showed a decrease in FT4 without changes in FT3 and TSH. Saegusa et al. (2010) and Zamoner et al. (2006) found decreases in both T3 and T4 and increases in TSH using 0.02% MMI. The dose level in these studies and our high-dose group was the same. Despite the less body weight gain in the high-dose group indicative of rather severe hypothyroid state, we did not see reductions in FT3 as anticipated from other works with MMI and related goitrogen, PTU. The absence of effect on T3 may reflect the measurement of unbinding hormone concentration rather than total one. Animal strains, body size of mother rats, and intake of MMI-dissolved water may also contribute to the differences in thyroid hormone levels. The effects of perinatal hypothyroidism can be alleviated by administering T4, which facilitates normal development of the cochlear tectorial membrane and development of normal cochlear potentials. Normal auditory function is recovered in hypothyroid animals treated with T4 (Uziel et al., 1985; Uziel et al., 1980). Therefore, it is clear that T4 affects auditory system development. It is possible that T4 may be linked to the abnormalities observed in the high-dose group in this study.

In summary, the MMI high-dose group showed reduced percentage PPI and body weight gain that did not return to control levels over the 12-month study period. This group also displayed an excess reaction to auditory startle stimuli, and plasma

FT4 concentrations decreased. Taken together, we conclude that perinatal hypothyroidism causes irreversible damage to auditory function.

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Figure Captions

Fig. 1 Percent PPI in rats treated with methimazole from gestational day 15 to postnatal day 21. Panel (a) displays percent PPI at three prepulse (PP) intensities and panel (b) displays percent PPI at 1, 6, and 12 months of age. Male and female data were pooled, as the effect of sex was not significant. Data represent mean and standard error of mean. * $p < 0.05$ compared with both control and low-dose groups.

Fig. 2 Startle response amplitude in rats treated with methimazole from gestational day 15 to postnatal day 21. Data are mean and standard error. * $p < 0.05$ compared with both control and low-dose groups.

Fig. 3 Body weight gain in rats treated with methimazole from gestational day 15 to postnatal day 21. Panel (a) exhibits body weights in male and female rats and panel (b) exhibits body weights at 1, 6, and 12 months of age. Data are mean and standard error. * $p < 0.05$ compared with both control and low-dose groups.

Fig. 4 Thyroid hormone concentrations in plasma and body weight gain in rat pups treated with methimazole from gestational day 15 to postnatal day 21. Thyroid hormones and body weights were measured on postnatal day 21. Plasma concentrations of FT4, FT3, and TSH are displayed in panels (a), (b), and (c), respectively. Male and female data were pooled because the effect of sex was not significant. Body weights are shown in panel (d). Data are mean and standard error. * $p < 0.05$ compared to both control and low-dose groups. Numbers in parentheses indicate sample sizes. The sample size in each group was different because some pups were so small that we were unable to collect sufficient blood to determine FT3, FT4, and TSH levels. Additional pups were supplied for thyroid hormone determination.

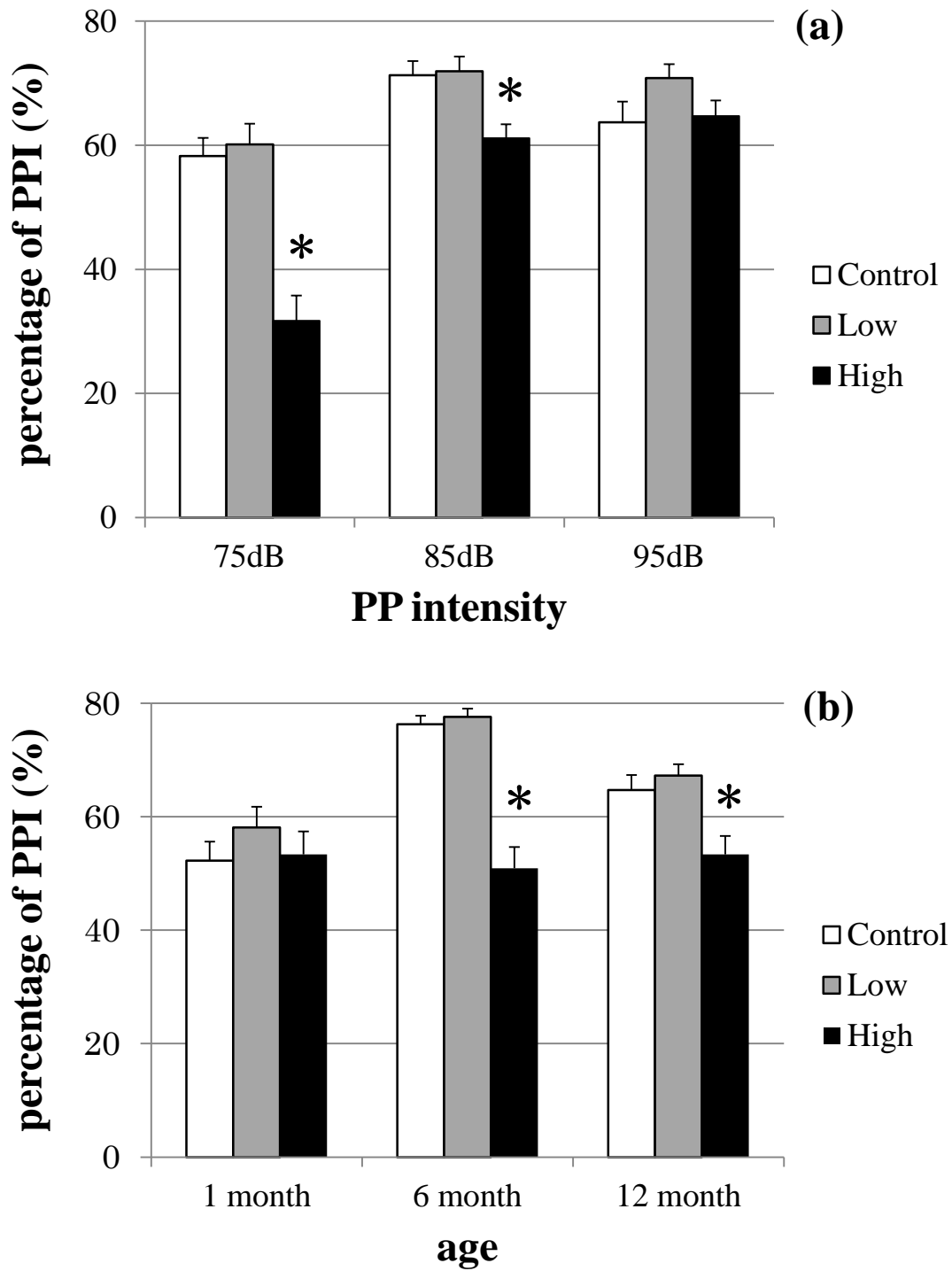


Fig. 1

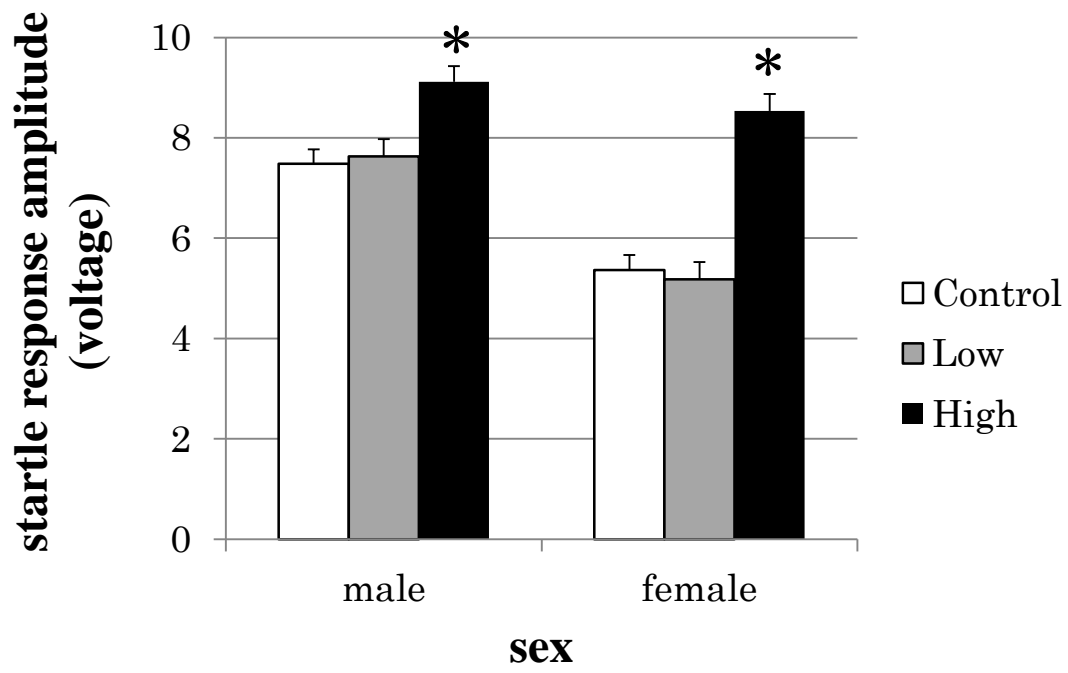


Fig. 2

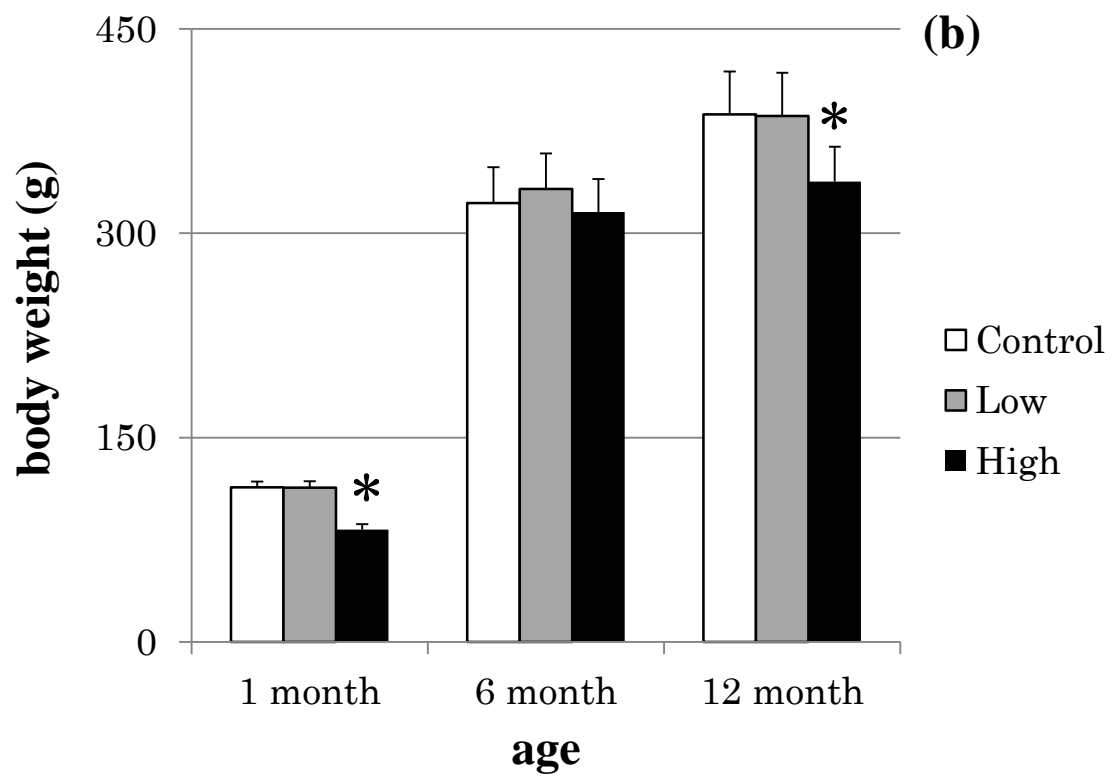
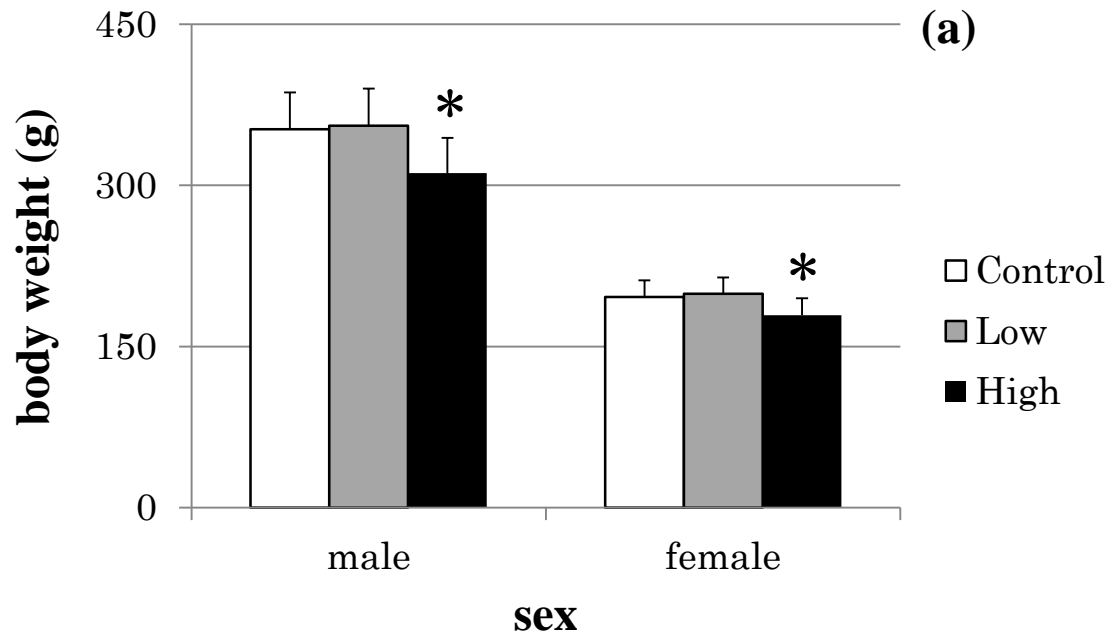


Fig. 3

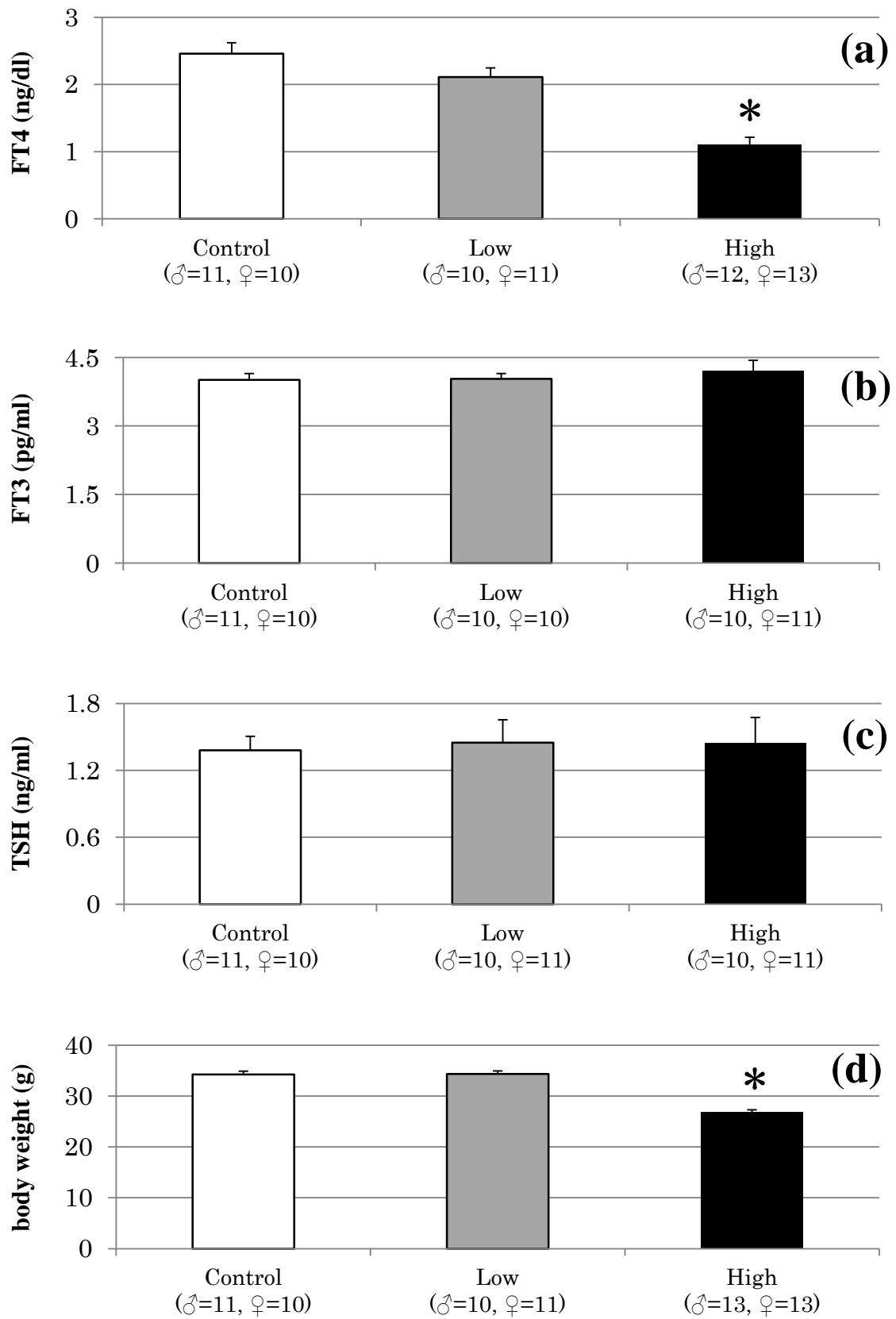


Fig. 4