

An Animal Model of Marginal Iodine Deficiency During Development: The Thyroid Axis and Neurodevelopmental Outcome*

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Thyroid hormones (THs) are essential for brain development, and iodine is required for TH synthesis. Environmental chemicals that perturb the thyroid axis result in modest reductions in TH, yet there is a paucity of data on the extent of neurological impairments associated with low-level TH disruption. This study examined the dose-response characteristics of marginal iodine deficiency (ID) on parameters of thyroid function and neurodevelopment. Diets deficient in iodine were prepared by adding 975, 200, 125, 25, or 0 µg/kg potassium iodate to the base casein diet to produce five nominal iodine levels ranging from ample (Diet 1: 1000 µg iodine/kg chow, D1) to deficient (Diet 5: 25 µg iodine/kg chow, D5). Female Long Evans rats were maintained on these diets beginning 7 weeks prior to breeding until the end of lactation. Dams were sacrificed on gestational days 16 and 20, or when pups were weaned on postnatal day (PN) 21. Fetal tissue was harvested from the dams, and pups were sacrificed on PN14 and PN21. Blood, thyroid gland, and brain were collected for analysis of iodine, TH, and TH precursors and metabolites. Serum and thyroid gland iodine and TH were reduced in animals receiving two diets that were most deficient in iodine. T4 was reduced in the fetal brain but was not altered in the neonatal brain. Neurobehavior, assessed by acoustic startle, water maze learning, and fear conditioning, was unchanged in adult offspring, but excitatory synaptic transmission was impaired in the dentate gyrus in animals receiving two diets that were most deficient in iodine. A 15% reduction in cortical T4 in the fetal brain was sufficient to induce permanent reductions in synaptic function in adults. These findings have implications for regulation of TH-disrupting chemicals and suggest that standard behavioral assays do not readily detect neurotoxicity induced by modest developmental TH disruption.

Key Words: thyroid; iodine; hypothyroidism; hippocampus; neurodevelopment; learning; synaptic function; biologically based dose-response models; BBDR.

Thyroid hormone (TH) is essential for normal brain development (Williams, 2008), so it is important to ensure that aspects of the human environment support adequate thyroid function. An important element of the human environment is nutritional status, and having adequate supplies of dietary iodine is key to thyroid health. Severe, chronic iodine deficiency (ID) during fetal and early neonatal development leads to hypothyroidism and mental retardation in children (DeLange, 1994; Zimmerman, 2007). A recent report indicated that in 2011 nearly one third of the global population had inadequate iodine intakes (Andersson *et al.*, 2012). Marginal ID is also associated with lower measures of cognitive function on a population level (Aghini Lombardi *et al.*, 1995; Laurberg, 2009) and has been found in more than 15% of women of child-bearing age in the United States (Hollowell and Haddow, 2007; Perrine *et al.*, 2010). This is alarming given that adequate iodine intake is crucial during gestation, when the mother is the only source of iodine for the fetus.

A number of thyroid-active environmental contaminants have been identified that interfere with the neuroendocrine system that controls the hypothalamic-pituitary-thyroid (HPT) axis (Brucker-Davis, 1998). It is highly likely that the impact of exposures to thyroid-disrupting chemicals is exacerbated under conditions of marginal ID (Kunisue *et al.*, 2010, 2011; Thilly *et al.*, 1993; van Wijk *et al.*, 2008). As such, there is a need to

develop tools to aid in assessment of health risks associated with thyroid-active chemicals for sensitive subpopulations, particularly women with preexisting thyroid disease, women with iodine insufficiencies, and pregnant women and their progeny (Blount and Valentin-Blasini, 2006; Knudsen *et al.*, 2002; Parham *et al.*, 2012; Wise *et al.*, 2012). Biologically based dose-response (BBDR) models are one such tool that can be implemented to reduce uncertainties in assessment of risks when extrapolating data from rodents to humans. Mathematical models that describe the impact of environmental stressors on some aspects of thyroid function in the adult (McLanahan *et al.*, 2008; Merrill *et al.*, 2003) and developing rats exist (Clewel *et al.*, 2003), but these were not designed to be predictive of overt or neurodevelopmental effects of stressors. To exploit the potential of computational models fully and improve the reliability of their predictions, a more complete appreciation of the quantitative relationships along the entire continuum is needed—from environmental exposure to early upstream biological perturbations and subsequent downstream overt effects (Parham *et al.*, 2012; Wise *et al.*, 2012).

Specifically for models of developmental TH insufficiency, there exists considerable uncertainty in three main areas: (1) changes in the dynamics of thyroid economy during the different life stages, (2) dose-response relationships that extend to modest degrees of perturbation, and (3) determining the linkage between modest degrees of HPT axis perturbation and downstream neurological consequences. The current study was conducted to address some of these uncertainties. We report the effects of ID on different HPT axis parameters in pregnant rats, fetuses, and neonates. We also report the effects of relatively modest degrees of TH insufficiency on a number of neurological endpoints in the adult offspring. In a companion article, a quantitative BBDR model is described for neonatal rats, incorporating the critical biological parameters in the dam and neonate garnered from results of the present study (Fisher *et al.*, 2012).

MATERIALS AND METHODS

Animals

A total of 207 young adult female Long Evans rats, aged 40 to 60 days, were obtained from Charles River Breeding Laboratory (Raleigh, NC). In our

facility, animals were paired housed in polycarbonate cages on pine shaving bedding and permitted free access to food (Purina 5001 chow) and water. Animals were housed in a facility certified by Association for Assessment and Accreditation of Laboratory Animal Care, and experiments were conducted using the protocol approved by Institutional Animal Care and Use Committee. Temperature was maintained at 22°C, with relative humidity of 40–60% and a 12-h light:dark (7:00 a.m.:7:00 p.m.) photoperiod.

Iodide-deficient diets. After 2–4 weeks, animals were assigned to diet groups, counterbalanced for weight, and provided diets containing one of five iodine concentrations (Research Diets, Newark, NJ). The stock casein diet was based on nutritional standards for laboratory animal diets established by the American Institution of Nutrition (1977). With the exception of iodine content, this diet was nutritionally replete, and animals did not differ in body weight gain from similarly aged female rats of this strain maintained on Purina 5001 chow, as described by Gilbert *et al.* (2011). The iodine content of the five casein-based diets was varied by adjusting the quantity of potassium iodate added to the base AIN-76A stock diet, nominally ranging from ample (Base I + 0.975 µg/gm, D1) to deficient (Base I = 0.025 µg/gm, D5), as summarized in Table 1. D2 contains the recommended iodine for AIN-76A diet; D5 represents the stock AIN diet with no iodine added. D2 and D3 meet previously established minimal requirements sufficient to meet physiological requirements (3–5 µg I/day; Heninger and Albright, 1975). D1 is far in excess of them but comparable to standard Purina 5001 and 5008 rat chow routinely used in our facility for nonpregnant and pregnant rats (Table 1). Food consumption was monitored by weighing feed bins thrice weekly and calculating mean daily consumption per rat throughout the prebreeding, gestational, and lactational periods (Table 1). Body weights were measured weekly prior to breeding and daily during pregnancy. After parturition, pup and dam body weights were determined on postnatal day (PN) 4, PN14, and PN21.

Breeding. Animals were fed specialized diets for a minimum of 6.5 weeks prior to breeding. Vaginal smears were monitored for 3 weeks prior to breeding and receptive females placed with males overnight on the evening of proestrus. The following morning, successful copulation was evaluated by examining females for the presence of a vaginal plug and sperm in the vaginal fluid. If present, this was designated as gestational day (GD) 0. Thereafter, animals were single housed, and body weight was monitored daily.

Gestational study. One group of dams from the control (D1) and the two lowest iodine (D4 and D5) diets were sacrificed on GD16 or GD20. All animals were sacrificed by decapitation between 8:00 a.m. and 12:00 p.m., and to avoid a temporal bias in sample collection, each diet group was sampled consecutively until all animals were sacrificed. Dam and fetal trunk blood, thyroid gland, placenta, and amniotic fluid were collected from dams at sacrifice. Trunk blood from fetuses was pooled across each litter sacrificed on GD20 and placed on ice to clot before serum separation, as described below. It was not possible to collect sufficient volumes of blood for hormone analysis from the fetuses on GD16. The brains were removed from three to four fetuses per litter at each gestational time point and forebrains dissected. All tissues were flash frozen in liquid nitrogen and stored at –80°C until analysis.

TABLE 1
Mean (± SE) Daily Food Consumption at Each Phase of the Study and Calculated Mean Daily Intake of Iodine Across Diet Groups

Diet	Nominal ng I/g of chow	Measured ng I/g of chow	Prebreeding food intake (g/day)	Gestation food intake (g/day)	Lactation food intake (g/day)	Prebreed (µg I/day)	Gestation (µg I/day)	Lactation (µg I/day)
1	1000	1300	19.0 (± 0.60)	21.4 (± 1.16)	30.3 (± 2.05)	24.82	27.86	39.32
2	225	205	18.1 (± 0.40)	22.5 (± 0.94)	31.2 (± 2.27)	3.72	4.61	6.38
3	150	135	19.3 (± 0.70)	22.1 (± 1.10)	30.6 (± 1.83)	2.60	2.99	4.13
4	50	36	18.3 (± 0.58)	20.7 (± 1.26)	31.3 (± 2.95)	0.66	0.74	1.12
5	25	10	19.0 (± 0.84)	22.3 (± 1.21)	29.3 (± 2.44)	0.19	0.22	0.29

Notes. Potassium iodate was added to the base chow formulation in varying quantities. Measured iodide (I) was assessed by IC-MS-MS as iodate as previously reported (Gilbert *et al.*, 2011).

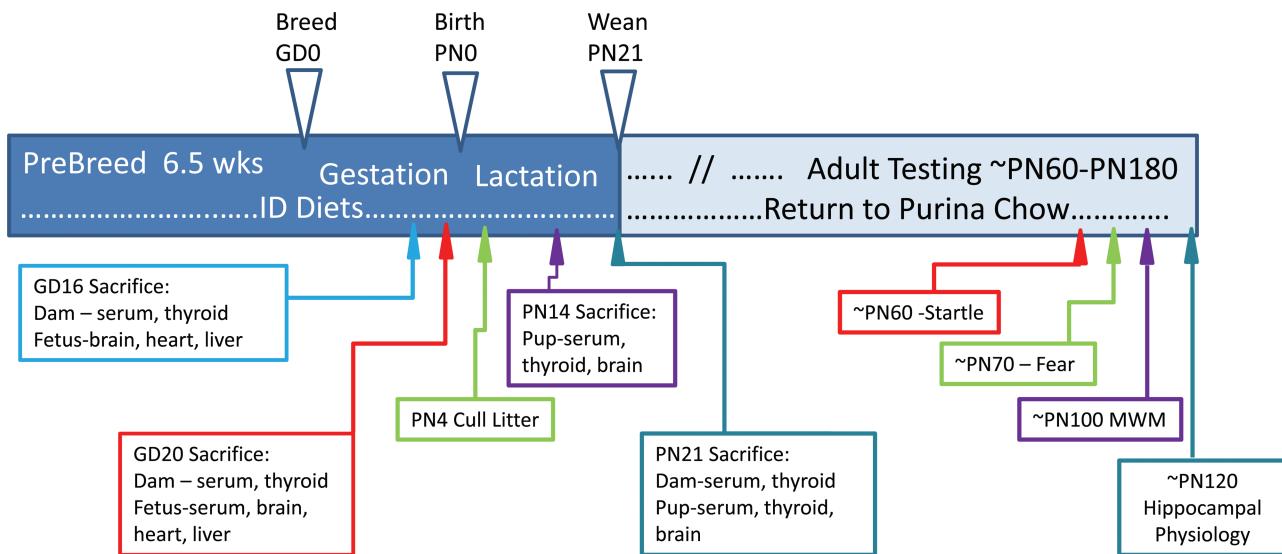


FIG. 1. Timeline of exposure and assessments. Female rats were placed on iodine-deficient diets for a minimum of 6.5 weeks before breeding and maintained on these diets throughout gestation and lactation. A subset of dams and fetuses were sacrificed before (GD16) and after (GD20) the onset of fetal thyroid function and a number of tissues collected. The remaining dams were allowed to give birth, and pups were harvested for tissue collection on PN14 and PN21. Dams were sacrificed when pups were weaned on PN21, and thyroid glands and serum were collected. At weaning, remaining pups were placed on standard laboratory chow and allowed to mature to adulthood. Different groups of animals were assessed on behavioral and neurophysiological assays beginning on ~PN60 with completion of all testing by ~PN180.

Postnatal study. Dams and their litters were maintained on the special iodine diets until pups were weaned on PN21. Litters were culled to a maximum of 10 pups per litter on PN4, balancing an equal number of males and females as much as possible. Trunk blood was collected from one male and one female pup per litter on PN14 and PN21. Dams were sacrificed on PN21, and trunk blood was collected. Thyroid glands were removed from dams and pups at sacrifice, weighed, and stored at -80°C for determination of iodide content and THs. Brains of PN14 and PN21 pups were removed; cortex, cerebellum, and hippocampus were dissected and immediately frozen in liquid nitrogen and stored at -80°C until analysis. All animals were sacrificed between 8:00 a.m. and 12:00 p.m., and time of sacrifice was counterbalanced across diet groups. At weaning, offspring were paired housed with same-sex litter mates and switched to Purina 5001 chow until behavioral and electrophysiological assessments were conducted. A summary and timeline of exposure and assessments performed are presented in Figure 1.

Chow and Serum Measures

Iodine was measured in each lot of chow prepared for these studies to verify the iodine content; these data were reported previously (Gilbert *et al.*, 2011). Serum perchlorate, thiocyanate, nitrate, and iodide were analyzed using previously published methods, with slight modifications (Valentin-Blasini *et al.*, 2007). Additional information on the aforementioned method and other analytical measures for serum and thyroid gland is available in Supplementary Materials.

Thyroid Gland Measures

Iodine from thyroid gland digests from postnatal dams and pups was analyzed using a previously published method for quantifying iodine in urine, with modification (Caldwell *et al.*, 2003), using inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS). This methodology quantifies all forms of iodide, iodine, and iodate as they are all converted into one form for analysis of total iodine. All molecular species are destroyed and all iodine converted to the I^- ion prior to quantification. Hormones and metabolites were measured in these samples according to procedures described by Kunisue *et al.* (2010, 2011), using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and an API 2000 electrospray triple quadrupole mass spectrometer. An high-performance liquid chromatography/inductively coupled plasma-mass

spectrometry (HPLC/ICP-MS) method was used to measure TH precursors (iodine, monoiodotyrosine [MIT], and diiodotyrosine [DIT]), hormones (3,5,3'-triodothyronine, T₃, and thyroxine, T₄), and metabolites (3,3'-diiodothyronine, T₂, and 3,3',5'-triodothyronine, rT₃) in the thyroid glands collected from pregnant dams on GD16 and GD20 according to previously published procedures (Tietge *et al.*, 2010).

Serum TH Analysis

Dams and pups. All blood samples were placed on ice to clot for 30 min, centrifuged to separate serum, and stored at -80°C until analysis. Serum concentrations of total T₄ and T₃ were measured in duplicate using standard solid-phase Coat-A-Count radioimmunoassay (RIA) kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA). Assay variation was assessed using the multivalent control module (Siemens Medical Solutions) to measure low, medium, and high total T₄ values before and after the experimental samples were analyzed by radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA). The lowest calibrator was 5 ng/ml for the T₄ and 10 ng/ml for the T₃ assays. The intra-assay coefficient of variations (CV) were 5.6 and 3.5% for T₄ and T₃, respectively, and interassay CVs were 5.8 and 5.1% for T₄ and T₃, respectively. Thyroid-stimulating hormone (TSH) was measured using a standard double-antibody assay as described by Thibodeaux *et al.* (2003). All samples were run in duplicate, and the intra-assay CV was 8.0%. Interassay variations ranged from 1 to 10%. The limits of detection for each assay, described as 90% assay binding, were 0.41 ng/dl for T₄, 14.3 ng/dl for T₃, and 0.39 ng/ml for TSH. With the exception of fetal serum (see below), all samples evaluated in this study were well above these detectable limits.

Fetal serum T₄ analysis. It was not possible to obtain sufficient volumes for serum TH assessments in GD16 fetuses. Blood from GD20 fetuses was pooled across the litter, and total T₄ was measured in 5 μ l volumes as previously described (Bansal *et al.*, 2005). Briefly, each assay tube contained 100 μ l barbital buffer (0.11M sodium barbital, 0.1% [wt/vol] 8-anilino-1-naphthalen-sulfonic acid sodium salt, 15% [wt/vol] bovine globulin, Cohn fraction II, 0.1% [wt/vol] gelatin [pH 8.6]), 100 μ l anti-T₄ (rabbit; Sigma) diluted to a final concentration of 1:30,000, and 100 μ l ¹²⁵I-labeled T₄ (12,000–15,000 cpm; PerkinElmer/NEN Life Science Products, Waltham, MA). Standards were prepared from T₄ (Sigma) measured using a Cahn electrobalance and were run in triplicate, whereas samples were run in duplicate. Standards were calibrated to measure

serum T4 levels from 0.4 to 25.6 µg/dl. Tubes were incubated at 37°C for 30 min and then chilled on wet ice for 30 min. Radioactivity was precipitated by adding 300 µl ice-cold polyethylene glycol 8000 (20% [wt/vol]; Sigma). Tubes were centrifuged at 1800 × g for 20 min at 4°C, the supernatant was aspirated, and the pellets were counted in a γ-counter (Cobra II; PerkinElmer). The assay was run at 40–50% binding; nonspecific binding was generally less than 8%.

Brain Hormone Analysis

Cortex samples from fetal brains on GD16 and GD20 and from PN14 pups from each litter were assessed for tissue concentration of T4 using radioimmunoassay according to procedures previously described (Bansal *et al.*, 2005). Briefly, tissue samples (~50 mg) were homogenized in cold methanol containing 25 µM 2-iodopropanoic acid and 1.17 mM propylthiouracil (PTU). The homogenate was spiked with ¹²⁵I-T4 and centrifuged. The resulting supernatant was transferred to a fresh 1.5-ml tube and evaporated to dryness using a speed vacuum. A volume of 500 µl of cell lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% NP40, 1× protease inhibitor cocktail) was added to the cell pellet for total protein analysis (BCA Protein Assay kit, Pierce). Dried tissue extracts were resuspended in 50 µl cold methanol and extracted with 200 µl chloroform and 50 µl barbital buffer as described above. The aqueous phase was removed, dried in a speed vacuum, counted for extraction efficiency calculations, and stored for T4 analysis as described above.

Behavioral Tests of Altered Neurodevelopment

A series of behavioral tests were performed to evaluate developmental neurotoxicity in adult male and female offspring. No treatment-related effects were observed in acoustic startle response, Morris water maze performance, or trace fear conditioning; these methods and results (Figs. 5–7) are summarized in Supplementary Material.

Synaptic Transmission in Dentate Gyrus

Surgery. Adult male offspring (4–6 months of age) selected from litters (1–2/litter) from each of three diet groups (D2, D4, and D5) were anesthetized with urethane (1.5–2 gm/kg, ip) and prepared for surgical implantation of electrodes as previously described (Gilbert, 2011). Two animals were assessed each day, and dose groups were counterbalanced over days to equate the mean age across groups. Data represent 11 D2, 13 D4, and 21 D5 animals sampled from 8, 9, and 12 litters, respectively.

Animals were mounted in a stereotaxic frame, and bipolar twisted stainless steel wire electrodes (250 µm in diameter, insulated except for the cut tips, and crimped onto gold-plated Amphenol pins) were lowered into the angular bundle of the perforant path according to flat skull stereotaxic coordinates (7.2 mm posterior to bregma, 4.1 mm lateral to the midline). A monopolar tungsten wire recording electrode was lowered into the ipsilateral dentate gyrus 3.5 mm posterior to bregma and 2–2.2 mm lateral to the midline. Nominal depths for stimulating and recording electrodes were 2.2 and 3.5 mm below dura, respectively, but optimal depth placement was achieved through electrophysiological monitoring of the response evoked in the dentate gyrus following single-pulse perforant path stimulation.

Stimulation of the perforant path evokes a monosynaptic extracellular field potential that can be reliably recorded from electrodes placed in the hilar region of the dentate gyrus. The field potential comprised an initial positive component, the excitatory postsynaptic potential (EPSP), and a negative compound action potential, the population spike (PS) (see insets of Fig. 10C). The positive component provides an index of synaptic activity comprising the summed EPSPs at the level of the dendrites. The slope of the EPSP was calculated as the rate of amplitude change for the initial positive component of the dentate gyrus field potential prior to PS onset. The EPSP peak was taken as the most positive peak on the waveform. The PS was derived from the amplitude of a line connecting the lowest value of the negative potential to the point of intersection of a tangent connecting the two positive peaks of the potential.

Excitatory and inhibitory synaptic function. Once optimal electrode placement based on response morphology and amplitude was achieved, responses

evoked by single-pulse stimulation of the perforant path (biphasic square wave pulses, 0.1 ms duration using a Grass S-88 stimulator, and PSIU-6 constant current converters) were monitored at 15-min intervals for a minimum of 1 h to ensure stability prior to commencement of formal testing (Gilbert and Mack, 1999). Responses were amplified, digitized (33 kHz sampling rate), averaged using LabWindows (National Instruments, Austin, TX) and custom designed software, and stored on a PC for later analysis. Upon stability, typically 2–3 h after initial electrode placement, paired pulses were used to probe inhibitory synaptic transmission. Two pulses of equal stimulus intensity were delivered at interpulse intervals (IPI) of 10, 20, 30, 70, and 250 ms at a maximal stimulus intensity of 1500 µA (100%), and those yielding PS amplitudes 50 and 20% of maximum PS amplitude, independently selected for each individual animal. Ten pulse pairs were averaged at each IPI and each intensity. Data were expressed as a ratio of test pulse (pulse 2) to conditioning pulse (pulse 1) PS amplitude. Excitatory synaptic transmission was evaluated by administering a series of 25 intensities ranging from 50 to 1500 µA (base to peak), with 5 pulses/intensity averaged and 10 s between each pulse. This comprised the input/output (I/O) function upon which differences in baseline synaptic transmission were assessed, as well as the reference I/O to determine the induction of long-term synaptic plasticity (i.e., pretrain I/O) following administration of theta-burst stimulation.

Long-term potentiation. Immediately following the collection of the pretrain I/O, five averages (5 sweeps/average) were collected at 5-min intervals at a midrange stimulus intensity (probe stimulus) selected to produce a response at 50% of maximal PS in the pretrain I/O functions. Averages of 10 pulses were sampled again 1, 15, 30, 45, and 60 min after delivery of long-term potentiation (LTP)-inducing trains to assess the magnitude of synaptic potentiation within the immediate posttrain period. LTP was induced by delivering three train pairs (i.e., two 1500 µA 4-pulse bursts at 400 Hz with a 200-ms interval between each burst, repeated thrice at 10-s intervals). A second I/O function was collected 1 h after train delivery upon completion of probe stimulus sampling at the 60-min time point. LTP magnitude was determined by the change in EPSP and PS amplitude of the probe stimulus response before after train delivery as well as a comparison of these parameters in pre- and posttrain I/O functions.

Statistical Analysis

All data were subjected to ANOVA using Statistical Analysis Software (SAS, Cary, NC). Repeated-measures ANOVAs were utilized for body weight, food consumption, and electrophysiological measures of synaptic transmission. Litter was the unit of analysis. For electrophysiological assessments in which more than one animal from a single litter was evaluated, the mean across litter was calculated, and this value was entered into analysis. One-way ANOVAs were used to evaluate THs, thyroid weights, serum, and thyroid gland analytes. When significant main effects or interactions were achieved, group differences were evaluated using Dunnett's *t*-test with *p* value set at 0.05.

RESULTS

General Toxicity Measures

Food intake dropped from a mean of 24 g/day at the beginning of casein diet to 16 g/day during weeks 3 through 6, but no differences were seen as a function of iodine content in the diet (Supplementary fig. 1A). As expected, daily food intake increased during pregnancy to ~25 g/day just prior to parturition, and no differences were seen as a function of diet group (Supplementary fig. 1B). Food consumption of dams rose again during lactation to a mean consumption of 35 g/day by lactational day 10 and a further increase to ~45 g/day for the dam and her litter at weaning on PN21 (Supplementary fig. 1C). To calculate daily iodine intake, mean values across these three phases of exposure were calculated for each diet condition and

multiplied by the average content of iodine per gram of chow and are summarized in [Table 1](#).

Dams gained weight during prebreeding ([Supplementary fig. 2A](#)) and gestation ([Supplementary fig. 2B](#)) as expected, and no diet-related differences were detected. Body weights measured postnatally in dam and offspring did not differ across diet groups ([Supplementary figs. 3A–C](#)). These data demonstrate comparable nutritional status of dams and pups across varying degrees of dietary ID at all phases of the life cycle examined.

A large number of measures were taken at various ages in different life stages. A summary table of the bulk of these measures indicating where differences from control diets were observed is presented in [Table 2](#). Although such a summary does not lend itself to specifics on doses, the bulk of the changes when observed were limited to one or both of the most iodine-deficient diets.

Analytes in Serum and Thyroid Gland

Iodide in serum. Iodide was measured in the serum of fetuses, dams, and pups ([Fig. 2](#)). As expected, dose-dependent reductions in serum concentrations of iodine were seen. The highest levels of serum iodine were seen in nursing pups of D1. Serum concentrations of perchlorate, thiocyanate, and nitrate were evaluated because they may each interfere with the Na^+/I^- symporter (NIS) ([Di Bernardo et al., 2011; Dohán et al., 2007](#)). With minor exceptions, the mean values were low and constant across all diet groups and ages (perchlorate $< 0.36 \text{ ng/ml}$, nitrate $< 5 \mu\text{g/ml}$, thiocyanate $< 10 \mu\text{g/ml}$ [[Supplementary Table 1](#)]).

Thyroid gland weights. The average weight of the thyroid gland provides a crude measure of thyroid gland hypertrophy in response to falling levels of serum TH. Under control conditions, thyroid gland weight of dams was higher in lactating (~25 mg) than in pregnant dams (~18 mg) ([Fig. 3A](#)), and thyroid gland weights were lower in pups than in adults ([Fig. 3B](#)). Increases in thyroid gland weight were observed in dams from D4 and D5 on GD16 and GD20 and at the end of lactation and were statistically significant in pregnant dams. Significant increases in the thyroid gland weight of pups were also evident and limited to the most deficient diet on PN21. Smaller increases in PN14 pups did not reach statistical significance.

Thyroid gland iodine and hormones. [Figure 4](#) presents serum iodide for all groups as a function of dietary iodine and suggests that lactating dams exhibit a greater degree of serum iodide depletion than other groups ([Fig. 4A](#)). Drops in serum iodide were associated with lower concentrations of thyroidal iodine ([Fig. 4B](#)), T4 ([Fig. 4C](#)), and T3 ([Fig. 4D](#)). Mean ($\pm \text{SEM}$) concentrations of iodine, T3, and T4 within the thyroid gland of dams from which these plots were derived can be found in [Supplementary figure 4](#). Briefly, the concentration of T3 in the thyroid gland was marginally reduced in D4 dams but with greater drops evident with greater ID of D5 ([Supplementary fig. 4C](#)). Thyroidal T4 was reduced by 50–75% in the dams of D4 and D5 during gestation, with further declines evident at the

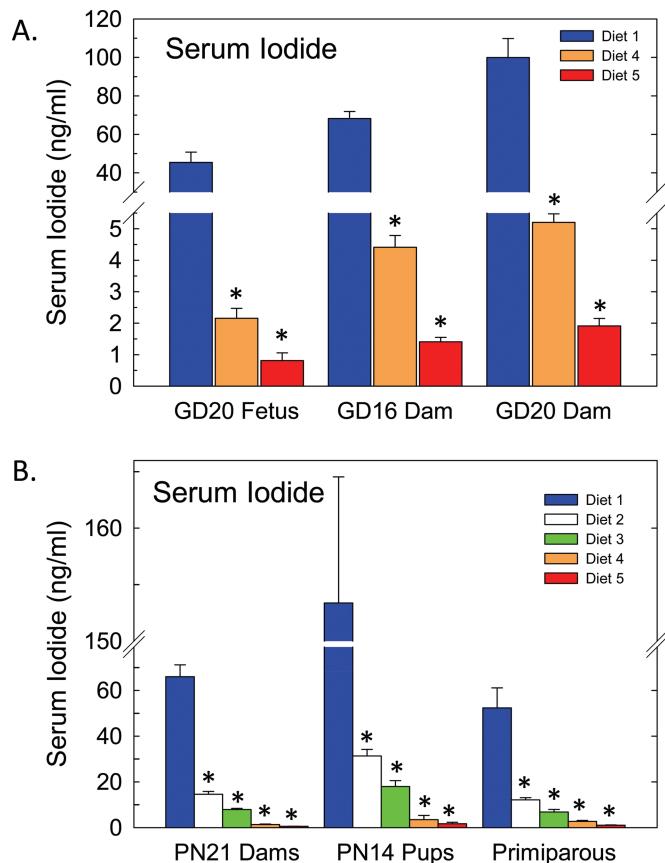


FIG. 2. Serum iodide after dietary ID. Mean ($\pm \text{SEM}$) iodide in serum in pregnant dam, in late-stage fetus (A), and in lactating dam, pup, and nonpregnant female (B) in response to dietary ID. Diet-dependent reductions in serum concentrations of iodine were seen in all groups. The relationship of dietary and serum iodide as a function of life stage may be better appreciated in Figure 6A.

end of lactation (75–90% declines in D4 and D5; [Supplementary fig. 4B](#)). Pup thyroid gland concentrations of T3 and T4 were reduced to a similar degree as postnatal dams (data not shown) but demonstrate a higher sensitivity than dams when data are expressed as a function of serum iodide ([Figs. 4C and D](#)).

Under conditions of mild TH insufficiency, a preferential shift occurs to augment the relative synthesis and release of T3 over T4, representing an autoregulatory adaptive response of the thyroid gland that is independent of serum TSH ([Pedraza et al., 2006](#)). This autoregulatory shift is reflected as an increase in T3/T4 ratios in thyroid gland and serum. In this study, increased T3/T4 ratios, were more dramatic in the thyroid gland than in the serum, and were more exaggerated in the lactating dam and preweaning pup in the most deficient diet compared with the pregnant dam ([Table 3](#)). Increased thyroid gland T3/T4 ratios of GD16 and GD20 dams are comparable to those reported by [Pedraza et al. \(2006\)](#) in nonpregnant female rats at similar levels of ID in the gland (i.e., 150 and 275%) and the serum (200 and 250%). In our most deficient diet, much higher T3/T4 ratios were seen in the thyroid gland for lactating

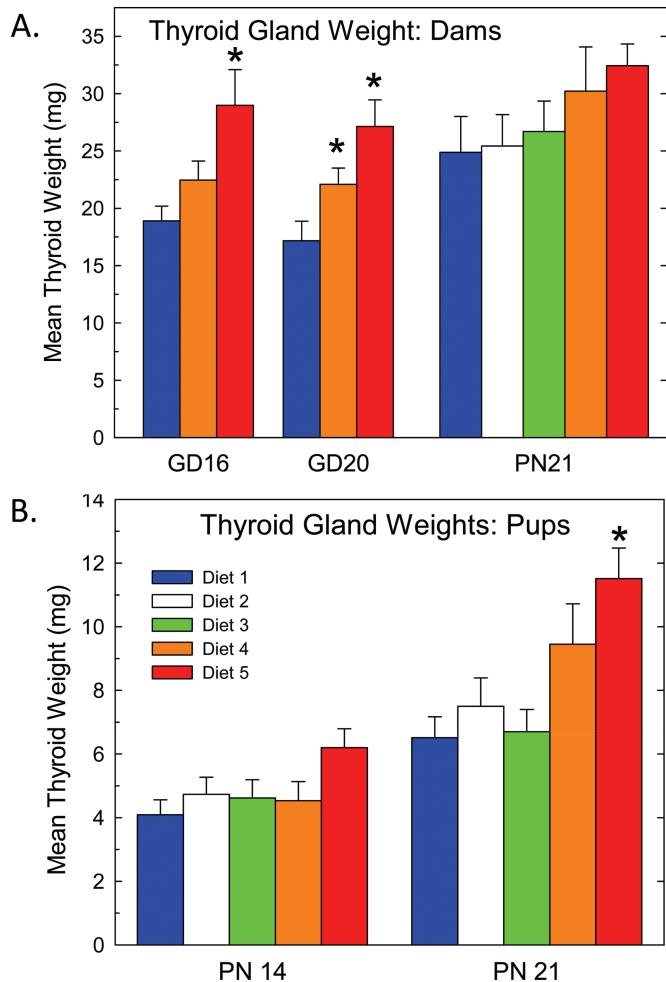


FIG. 3. Thyroid gland weight after dietary ID. Mean (\pm SEM) thyroid gland weight in pregnant and lactating dams (A) and pups on PN14 and PN21 (B). Significant increases in thyroid weight were detected only in pregnant dams (GD16 $F(2,25) = 6.08$, $p < 0.007$; GD20 $F(2,26) = 7.11$, $p < 0.0034$) but not in lactating dams ($F(4,64) = 1.53$, $p > 0.20$). Pups' thyroid weights were significantly increased on PN21 ($F(4,59) = 5.41$, $p < 0.0009$) but not on PN14 ($F(4,54) = 2.22$, $p > 0.08$). Increases when present were limited to the most deficient diet in most cases. Asterisks indicate significant difference from D1 using Dunnett's t -test, $p < 0.05$.

dams (525%) and PN14 pups (548%). These differences may reflect upregulation of autoregulatory responses in the thyroid gland in response to the higher iodine demands of the lactating dam and the developing pup. The augmentation in T3 synthesis and release from the thyroid gland during postnatal period contributes to maintenance of normal levels of serum T3 at the expense of T4 in serum (see below).

Thyroid gland hormone precursors and metabolites. The thyroid glands of dams sacrificed on GD16 and GD20 were also assayed for hormone precursors MIT and DIT and for hormone metabolites T2 and rT3 (Fig. 4). Thyroidal content of these compounds did not differ between control dams sacrificed at different gestational ages. The precursors MIT and DIT were reduced in a dose-dependent manner, the degree of

loss more extreme for DIT (~90% reduction in D5) than for MIT (60–70% in D5), with similar declines at both ages (all $p < 0.001$). The metabolite T2 was also suppressed severely (50 and 90%) by D4 and D5, respectively, and comparably across the two gestational time points. rT3 was not altered by D4 but reduced by 50% in the most deficient diet on GD16. A similar but nonsignificant decline was detected in GD20 (Fig. 5D) and PN21 (Supplementary fig. 4) dam thyroid glands.

Serum THs

Serum hormones in the fetus, pup, and pregnant and lactating dam are summarized in Figures 5 and 7. No changes in serum T3 were detected in any group (Figs. 6A and 7A; all $p > 0.18$). Reductions in serum T4 were observed in one or both of the two most deficient diets in all groups. Dam's T4 on GD16 was significantly reduced in the most deficient diet and in both iodine-deficient diets on GD20. Postnatally, significant declines in dam's T4 were limited to the most deficient diet ($p < 0.0001$). In the fetus, serum T4 was significantly reduced for both iodine-deficient diets assessed; on PN14 significant reductions were seen only in the most iodine-deficient diet, and on PN21 slight declines were evident in D4 and larger decrements seen in D5.

In general, the increases in serum TSH were small in magnitude (an approximate doubling in postnatal dams) and, for the most part, limited to the most deficient diet. No effect on serum TSH was seen in GD16 dams, and small increments were detected on GD20 that increased further in the lactating dam. No changes in serum TSH were evident in dams on GD16 despite a 50% reduction in circulating levels of T4. Although a marginally significant increase in TSH was detected in the serum on PN14 pups ($p < 0.049$), mean contrast tests did not show a significant increase in iodine-deficient pups. By PN21, larger and more consistent increases in serum TSH were evident, with significant increases detected in D5 pups ($p < 0.0001$). However, even the largest increases reported here were small relative to other hypothyroid treatments and ID manipulations (Gilbert and Sui, 2006; Pedraza *et al.*, 2006).

The relationship between thyroidal iodine and serum iodide content and serum T4 and TSH is summarized in Figure 8. Pregnant dams and pups were the most sensitive, displaying greater reductions in serum T4 and less severe reductions in serum iodide concentrations than the late-gestation fetus or the lactating dam (Fig. 8A). However, during pregnancy, serum T4 was reduced despite a higher thyroidal iodine content relative to the postnatal dam or pup (Fig. 8B). TSH was only marginally increased in dams and pups at weaning. The relationship with serum TSH reveals significant increases in serum TSH in late-gestation dams at low serum iodide (Fig. 8C) and thyroidal iodine (Fig. 8D) concentrations.

THs in Brain

The content of T4 was assessed using RIAs in brain tissue collected from GD16 and GD20 fetuses and pups on PN14

A: Iodine in Chow – Relationship to Iodide in Serum
B-D: Iodide in Serum- Relationship to Iodine, T3, T4 in Thyroid Gland

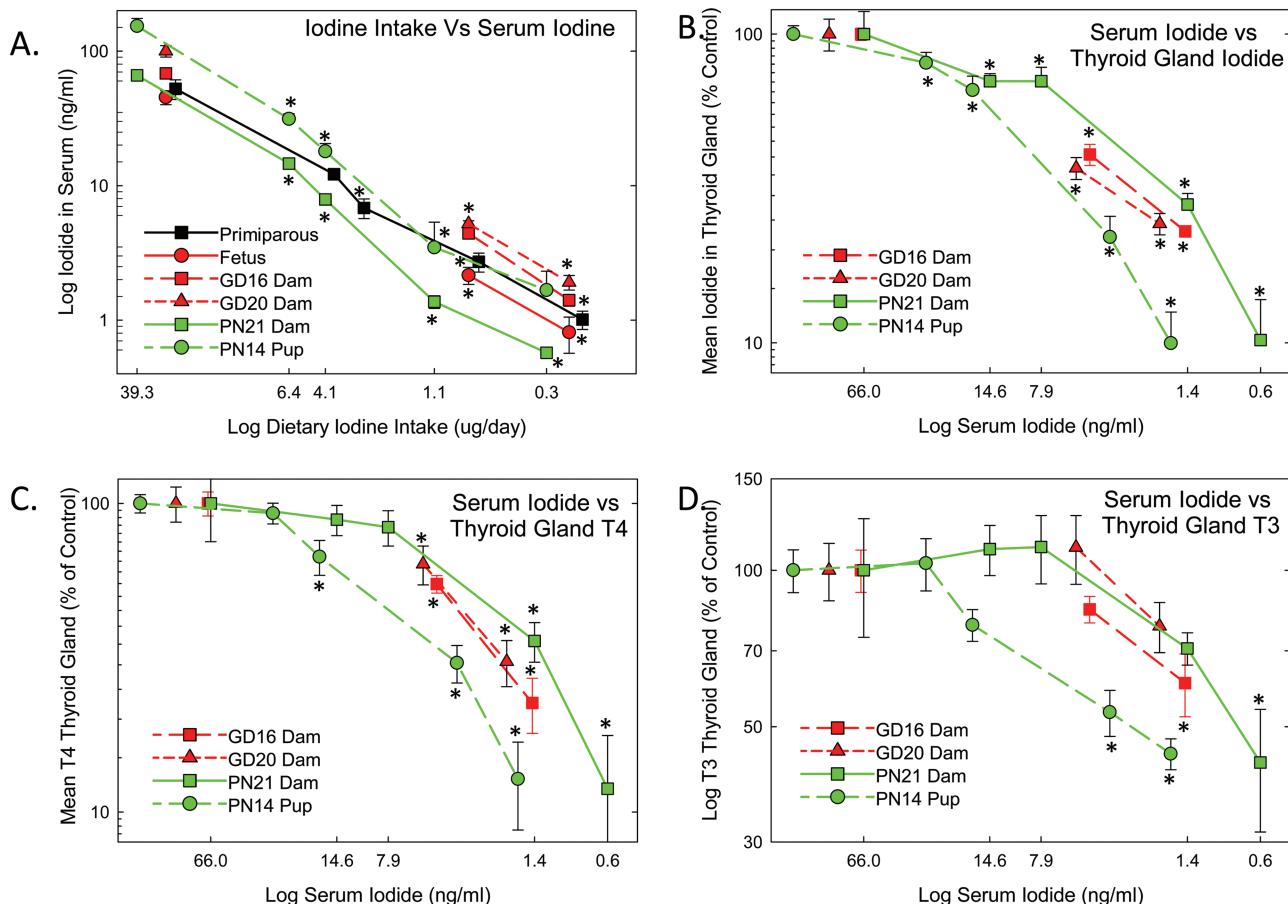


FIG. 4. Relating serum iodide and hormones within the thyroid gland. Mean (\pm SEM) iodide or iodine and THs in chow, serum, or thyroid gland. Asterisks represent significant differences from D1, with more than ample supplementation of iodine to the chow using Dunnett's mean contrast test following a significant main effect of diet in ANOVA. SEMs that are not apparent are smaller than the figure symbols. (A) Relationship of dietary iodine ($\mu\text{g}/\text{day}$) to serum iodide (ng/ml) in dam, fetus, neonate, and nonpregnant adult female rat. The lactating dam (PN21 dam) appears to be more sensitive to dietary iodine insufficiency with slightly lower levels of serum iodide than other groups at the same level of dietary intake. (B) Relationship of serum iodide (ng/ml) and thyroidal iodine expressed as a percent of control (D1). Neonates (PN14 pup) appear the most sensitive to lower iodine in the thyroid gland for a given level of serum iodide. (C) Relationship of serum iodide to thyroidal T4. As with thyroidal iodine stores, neonates (PN14 pup) appear to be more sensitive to thyroidal T4 stores than pregnant or lactating dams at lower levels of serum iodide. (D) Relationship of serum iodide to thyroidal T3. As seen in (C), neonates (PN14 pup) are more sensitive to declines in thyroidal T3 at any given level of serum iodide. Greater overall declines in T4 or T3 in the thyroid gland are consistent with greater percentage release of T4 over T3 into the serum (as gleaned from T3/T4 ratios, Table 3). Lactating dams from the most deficient diet also show greater declines than dams during pregnancy and fall to a plateau equivalent with the neonate for thyroidal iodine, T3, and T4 and may reflect time on diet in addition to sensitive life stage.

(Fig. 9). Cortical T4 levels dramatically increased with age (200 and 500% above GD16 levels at GD20 and PN14, respectively). Both iodine-deficient diets reduced cortical T4 in the fetus. Significant and dose-dependent reductions in cortical T4 were observed in the brains of the GD20 fetus ($p < 0.0034$), and mean contrast tests revealed significant declines in both iodine-deficient diets tested. Decrements were also detected in the GD16 fetal brain, but the control levels were more variable and the statistical analysis fell just short of statistical significance ($p > 0.06$). In contrast, no reliable differences in T4 were detected in the cortex of the PN14 pup ($p > 0.25$). The

relationship of serum T4 to fetal brain T4 is summarized in Figure 9C in which the sensitivity based on serum T4 appears greatest in GD20 fetus, followed by GD20 dam > PN14 pup > PN21 dam. The GD16 dam serum T4 appears the least sensitive to fetal cortex T4 reductions. Significant reductions in cortical T4 were seen at serum T4 reductions in dams of ~20%.

Synaptic Transmission and Plasticity in the Dentate Gyrus of Adult Offspring

Inhibitory synaptic transmission. Hippocampal field potentials were examined in male offspring from one iodine-sufficient

TABLE 2
Summary of Results of Basic Parameters Assessed Across Age and Condition

Body		Serum			Thyroid gland			Brain function							
Food intake	Body weight	Iodine	T3	T4	TSH	Thyroid weight	Iodine	T3	T4	Auditory sensory gating	Trace fear learning	Morris water maze	Inhibitory synaptic function	Excitatory synaptic function	Synaptic plasticity
GD16 Dam	—	—	↓	—	↓	—	↑	↓	↓	n/a					
GD20 Dam	—	—	↓	—	↓	↑	↑	↓	↓						
GD16 Fetus	n/a	nt	nt	nt	↓	nt	nt	nt	nt						
GD20 Fetus	n/a	nt	nt	nt	↓	nt	nt	nt	nt						
PN14 Pup	—	—	↓	—	↓	—	—	↓	↓						
PN21 Pup	—	—	nt	—	↓	↑	↑	nt	nt						
PN21 Dam	—	—	↓	—	↓	↑	—	—	↓						
PN60+ Pup	n/a	—	nt	—	—	—	nt	nt	nt	—	—	—	—	↓	—

Notes. Arrows indicate either statistically significant increases or reductions in parameter in the most deficient iodine diet (Diet 5). — indicates no statistically reliable change in that parameter was detected; n/a, not applicable; nt, not tested.

TABLE 3
Thyroid Gland and Serum T3/T4 Ratios (Mean \pm SEM) and Calculated Percent of Control Ratio

Diet	N	Gland T3/T4	Serum T3/T4	Gland, % control	Serum, % control
Dams					
GD16	1	10	0.22 \pm 0.100	3.26 \pm 0.36	100
GD16	4	10	0.35 \pm 0.014	3.65 \pm 0.29	160*
GD16	5	8	0.65 \pm 0.065	6.42 \pm 0.47	295*
GD20	1	10	0.21 \pm 0.029	3.73 \pm 0.27	100
GD20	4	10	0.41 \pm 0.026	4.89 \pm 0.25	196*
GD20	5	7	0.66 \pm 0.058	6.95 \pm 0.81	314*
PN21	1	4	0.16 \pm 0.009	2.34 \pm 0.19	100
PN21	2	5	0.19 \pm 0.014	2.76 \pm 0.27	124
PN21	3	5	0.20 \pm 0.009	2.60 \pm 0.22	130
PN21	4	5	0.32 \pm 0.031	3.57 \pm 0.42	206
PN21	5	5	0.82 \pm 0.161	6.45 \pm 0.75	525*
Pups					
PN14	1	4	0.11 \pm 0.005	2.07 \pm 0.09	100
PN14	2	5	0.12 \pm 0.007	1.82 \pm 0.11	110
PN14	3	4	0.14 \pm 0.013	2.03 \pm 0.12	121
PN14	4	5	0.20 \pm 0.017	2.37 \pm 0.11	183
PN14	5	5	0.61 \pm 0.212	4.47 \pm 0.81	548*
* <i>p</i> < 0.05 Dunnett's post hoc <i>t</i> -test after significant ANOVA on ratio values.					

(D2) and the two iodine-deficient (D4 and D5) diets to evaluate synaptic function. Assessments were performed well after return to euthyroid status, beginning ~4 months of age. Consistent with previous findings, depression of the PS elicited by the second pulse of the pair was seen at short IPIs (< 70 ms) that primarily reflect the activation of feedback GABA_A-mediated inhibition (Fig. 10). Augmentation of the population response to the second pulse of the pair was seen at intermediate intervals, followed by a second period of paired pulse depression at the longest interval tested. These phases are dominated by presynaptic potentiation and feed-forward inhibition, respectively. Clear experimental control over these physiological processes is evidenced by the expected intensity and IPI profiles displayed in Figure 10. No

evidence of any dietary-induced alteration in synaptic depression or facilitation was detected.

Excitatory synaptic transmission. I/O functions using a broad range of stimulus strengths were used to examine the integrity of excitatory synaptic function in adult offspring. As expected, I/O functions revealed that all groups demonstrated increases in synaptic response amplitude for EPSP slope, EPSP peak, and PS, with increasing stimulus intensity. However, the magnitude of excitatory synaptic transmission was reduced in diets D4 and D5. EPSP slope (Fig. 11A) and EPSP peak (Fig. 11B) were suppressed relative to controls and by a similar magnitude at both ID levels. A significant effect of diet ($F(2,26) = 5.08$, $p < 0.0137$) and diet \times intensity interactions ($F(48,624) = 9.32$, $p < 0.0001$) were observed for EPSP slope, and EPSP peak followed the same pattern (both $p < 0.01$). Mean contrast tests of each diet group I/O relative to control I/O indicated significant reductions at both dietary levels for both EPSP measures (all $p < 0.012$). PS amplitude (Fig. 11C) is a measure of neuronal excitability, the number of granule cells sufficiently depolarized by the EPSP to activate action potentials. This measure of baseline synaptic function was also reduced by ID. The overall effect of diet was not significant ($p > 0.45$), but significant diet \times intensity interaction was observed ($F(48,624) = 3.98$, $p < 0.0001$). Mean contrast tests by diet revealed significant reductions in PS amplitude for D4 and D5 (both $p < 0.0001$). Figure 11C depicts the relationship of cortical T4 in the GD20 fetus and reductions in synaptic transmission. Declines in fetal cortical T4 of ~15% were sufficient to impair synaptic function in the adult offspring.

Long-term potentiation. LTP is a model of synaptic plasticity that reflects the underlying mechanisms of memory at the synaptic level. It is induced by application of high-frequency stimulation to the hippocampal afferents and is assessed by the magnitude of increase in the EPSP and PS in

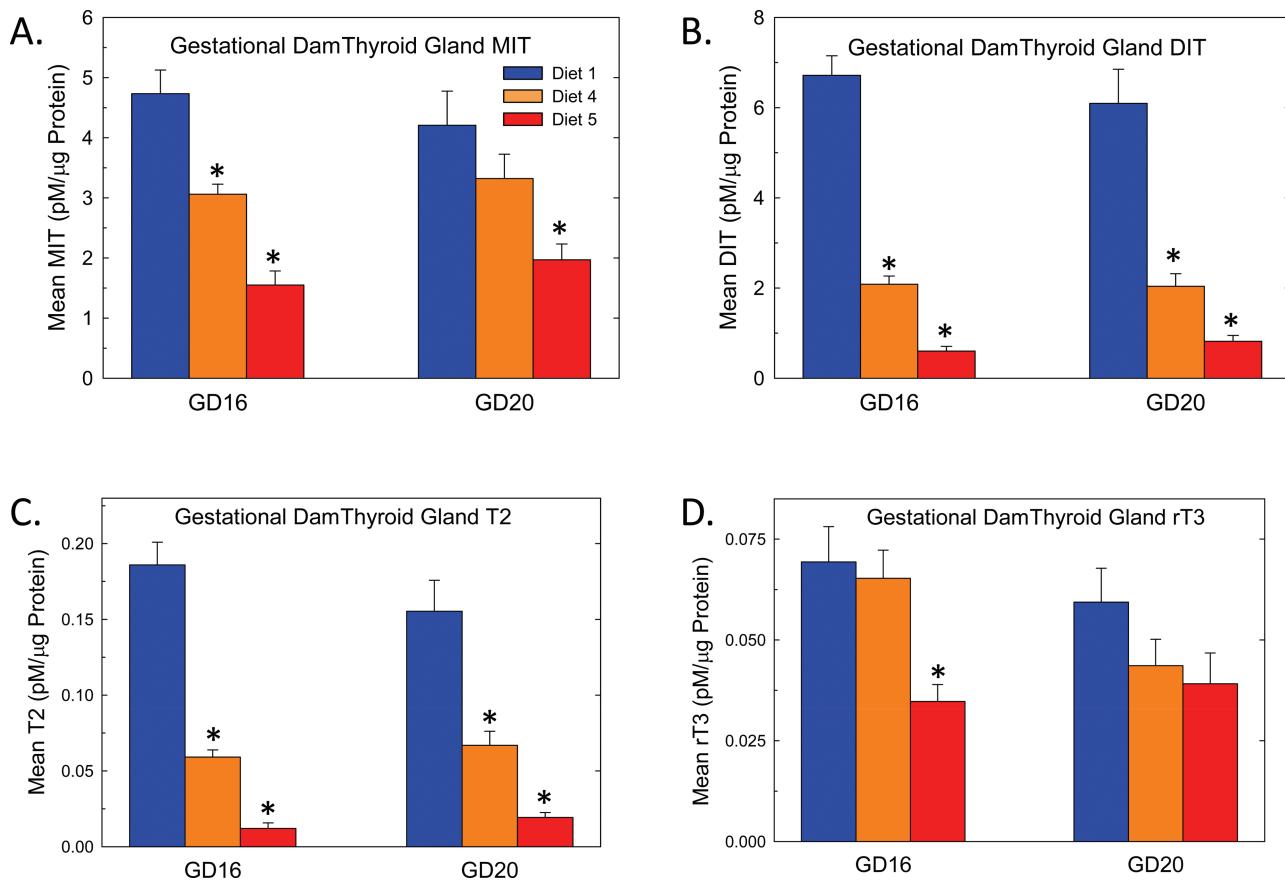


FIG. 5. Thyroidal levels of hormones, precursors, and metabolites. Mean (\pm SEM) TH precursors MIT, DIT, and the metabolites T2 and rT3 in the thyroid gland of the pregnant rat before (GD16) and after (GD20) the onset of fetal thyroid function. (A) Significant diet-dependent decreases were seen in MIT on GD16 ($F(2,25) = 29.12, p < 0.0001$) and GD20 ($F(2,24) = 5.29, p < 0.0125$) (A) and in DIT on GD16 ($F(2,25) = 115.12, p < 0.0001$) and GD20 ($F(2,24) = 27.7, p < 0.0001$) (B). The TH metabolite T2 was also significantly reduced on GD16 ($F(2,25) = 81.97, p < 0.0001$) and GD20 ($F(2,24) = 21.64, p < 0.0001$) (C). rT3 was also reduced in the most deficient diet on GD16 ($F(2,25) = 6.23, p < 0.007$) but not on GD20 ($F(2,24) = 1.92, p > 0.16$) (D) or in dams at weaning on PN21 ($F(4,19) = 2.81, p > 0.06$) (data not shown).

the dentate gyrus field potential. An increase in EPSP slope of the probe stimulus was maintained for 1 h after delivery of LTP-inducing trains, but no significant difference was seen in LTP magnitude across diets ($F(2,26) = 1.56, p > 0.23$). A significant decrease in the magnitude of PS LTP ($F(2,26) = 4.01, p < 0.03$) was observed, but the differences between diet groups were small (186, 158, and 178% for D2, D4, and D5, respectively) and not dose dependent. Consistent with analysis of probe stimulus change post LTP, an analysis of responses based on 20, 50, and 100% maximal PS responses in pre- versus posttrain I/O functions also failed to reveal any differences in EPSP or PS LTP across dietary groups (data not shown).

DISCUSSION

This study details the effects of responses to graded levels of dietary ID on the HPT axis in rats. In contrast to many existing reports, the absence of effects on dam or pup body

weight, food intake, and modest reductions in serum hormone signify that we achieved our goal of a model of ID that produced graded and moderate degrees of TH insufficiency. Serum, thyroid gland, and brain effects were observed in the two most deficient diets. No effects were seen on behavioral indices of sensory or cognitive function using components of a standard battery of tests used to detect developmental neurotoxicity. Persistent reductions in excitatory synaptic transmission were evident in three measures of dentate gyrus field potentials collected from adult offspring from the two most deficient diets despite provision of more-than-ample dietary iodine at weaning and return to euthyroid status thereafter. Very modest reductions in serum T4 (~20%) in the fetus, neonate, and pregnant or lactating dam were associated with measurable decrements in brain T4 in the fetus but not in the neonate. These small reductions in fetal brain T4 appear to be sufficient to alter the integrity of hippocampal synaptic function measured in the hippocampal dentate gyrus of the adult offspring.

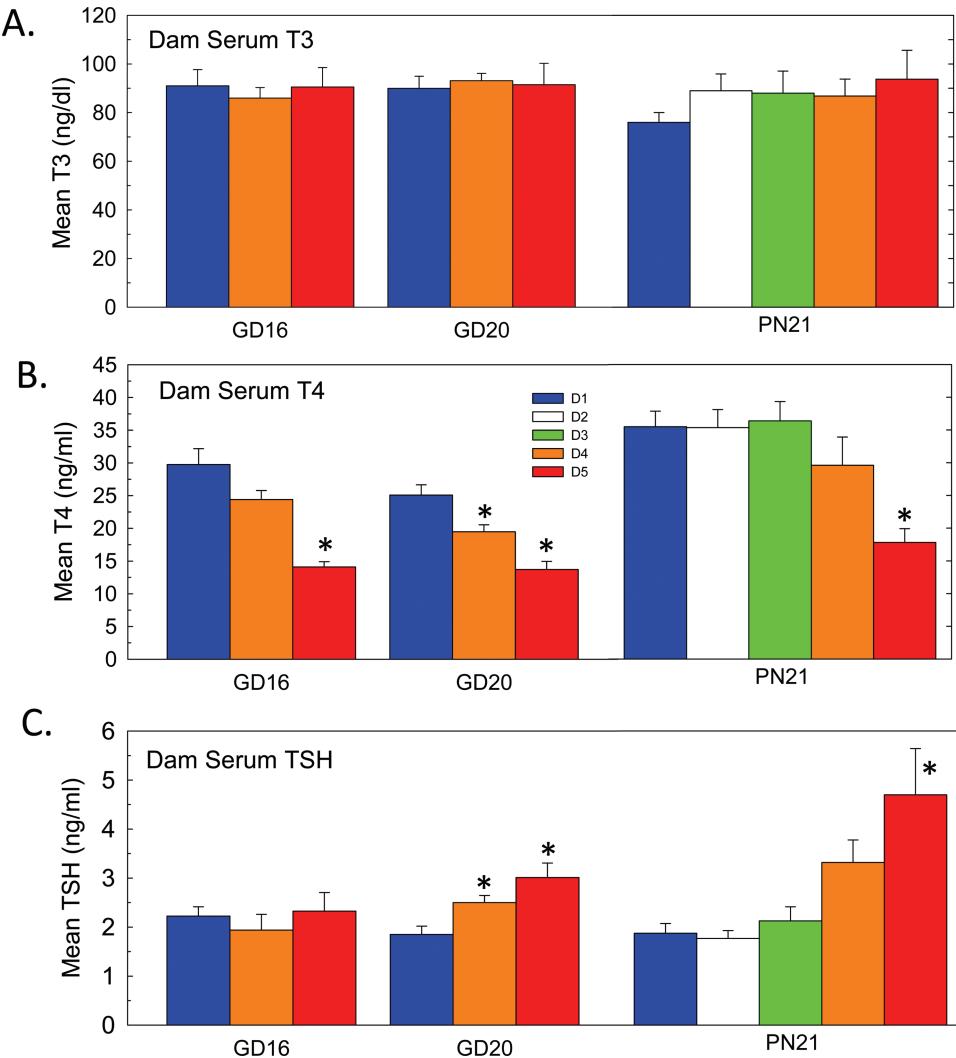


FIG. 6. Serum hormones in dams. Mean (\pm SEM) serum hormones in pregnant and lactating dams. (A) No detectable changes were seen in serum T3 in any diet group, D, or age (all $p > 0.25$). (B) Significant diet-dependent reductions were evident serum T4 from dams on GD16 ($F(2,25) = 18.8, p < 0.0001$), GD20 ($F(2,27) = 15.24, p < 0.0001$) and at weaning of pups on PN21 ($F(4,91) = 8.29, p < 0.0001$). (C) Serum TSH was marginally increased in dams in late (GD20 $F(2,26) = 8.66, p < 0.0013$) but not early gestation (GD16, $p > 0.63$). Postnatally, TSH increases were detected in dams (P21 $F(4,92) = 5.23, p < 0.0008$) and were limited to the most deficient diet group.

Model of Marginal ID

The maximal degree of serum hormone insufficiency attained in our study reflects hypothyroxinemia and not frank hypothyroidism, paralleling the “mild” and “moderate” ID conditions (1.0 and 0.5 μ g I/day) described by Pedraza *et al.* (2006) and Schröder-van der Elst *et al.* (2001). As the dietary manipulations did not alter food intake, weight gain, or serum T3, our findings are unconfounded by other nutritional insufficiencies or the presence of contaminants that may compete with iodine for thyroidal uptake. Our goal was to develop a model of marginal ID, and the findings stand in sharp contrast to recent reports of behavioral, molecular, and anatomical impairments accompanying developmental ID (Babu *et al.*, 2011; Dong *et al.*, 2005, 2011; Gong

et al., 2010a, b; Van Wijk *et al.*, 2008). With the exception of Babu *et al.* (2011), other studies indicated that significant reductions in body weight of dams and offspring, developmental delays, and dramatic reductions in TH indicative of severe hypothyroidism accompanied the dietary manipulation. Pretreatment or coadministration with perchlorate is a common practice in many studies (Escobar del Rey *et al.*, 1986, 1987; Kunisue *et al.*, 2011; Lavado-Autric *et al.*, 2003; Pedraza *et al.*, 2006; Schröder-van der Elst *et al.*, 2001; Van Wijk *et al.*, 2008). The presence of other contaminants that inhibit iodine uptake (e.g., thiocyanate, nitrate, perchlorate; see Blount and Valentin-Blasini, 2006; Dohán *et al.*, 2007) and nutritional or other mineral deficiencies (i.e., iron, copper, selenium, isoflavones; see Bastian *et al.*, 2010; Hess and

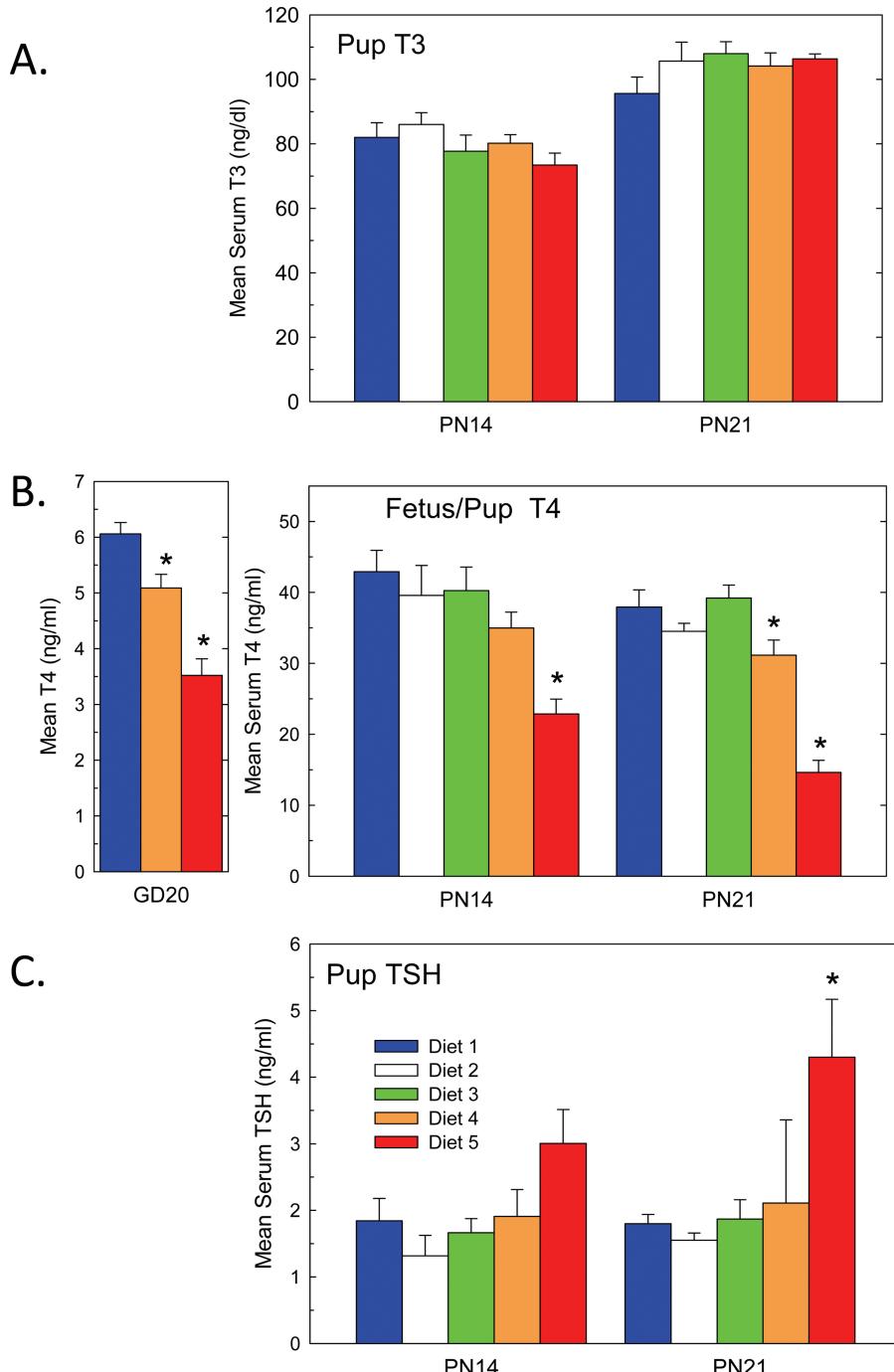


FIG. 7. Serum hormones in fetus and pup. (A) Mean (\pm SEM) serum T3 was not changed in the neonate (all $p > 0.25$). (B) Modest but significant declines in serum T4 were seen in fetus ($F(2,27) = 24.53, p < 0.0001$) and PN21 neonates ($F(4,26) = 13.43, p < 0.0001$) from the two most deficient diets. On PN14, typically the most sensitive postnatal age, significant reductions in serum T4 were limited to the most deficient diet ($F(4,58) = 30.72, p < 0.0001$). (C) Serum TSH increased marginally but significantly in PN14 ($F(4,21) = 2.85, p < 0.049$) and more robustly in PN21 ($F(4,53) = 7.29, p < 0.001$) pups from the most deficient diet. Asterisks represent significant difference from control using Dunnett's mean contrast *post hoc* test applied when a significant main effect of diet was detected in the main ANOVA. Serum T3 and TSH were not assessed in the fetus due to insufficient sample volumes.

Zimmermann, 2004; Schomburg, 2012; Zimmermann *et al.*, 2007) may contribute to a greater physiological compromise and more severe disturbance to the thyroid axis described by these authors. As such, the characteristic that distinguishes

this from previous neurodevelopmental studies is the severity of the iodine depletion and the consequent degree of TH disruption induced by the dietary manipulation. It is at these modest levels of thyroid disruption where information is

A C: Iodide in Serum- Relationship to Serum T4 and TSH
B D: Iodine in Gland - Relationship to Serum T4 and TSH

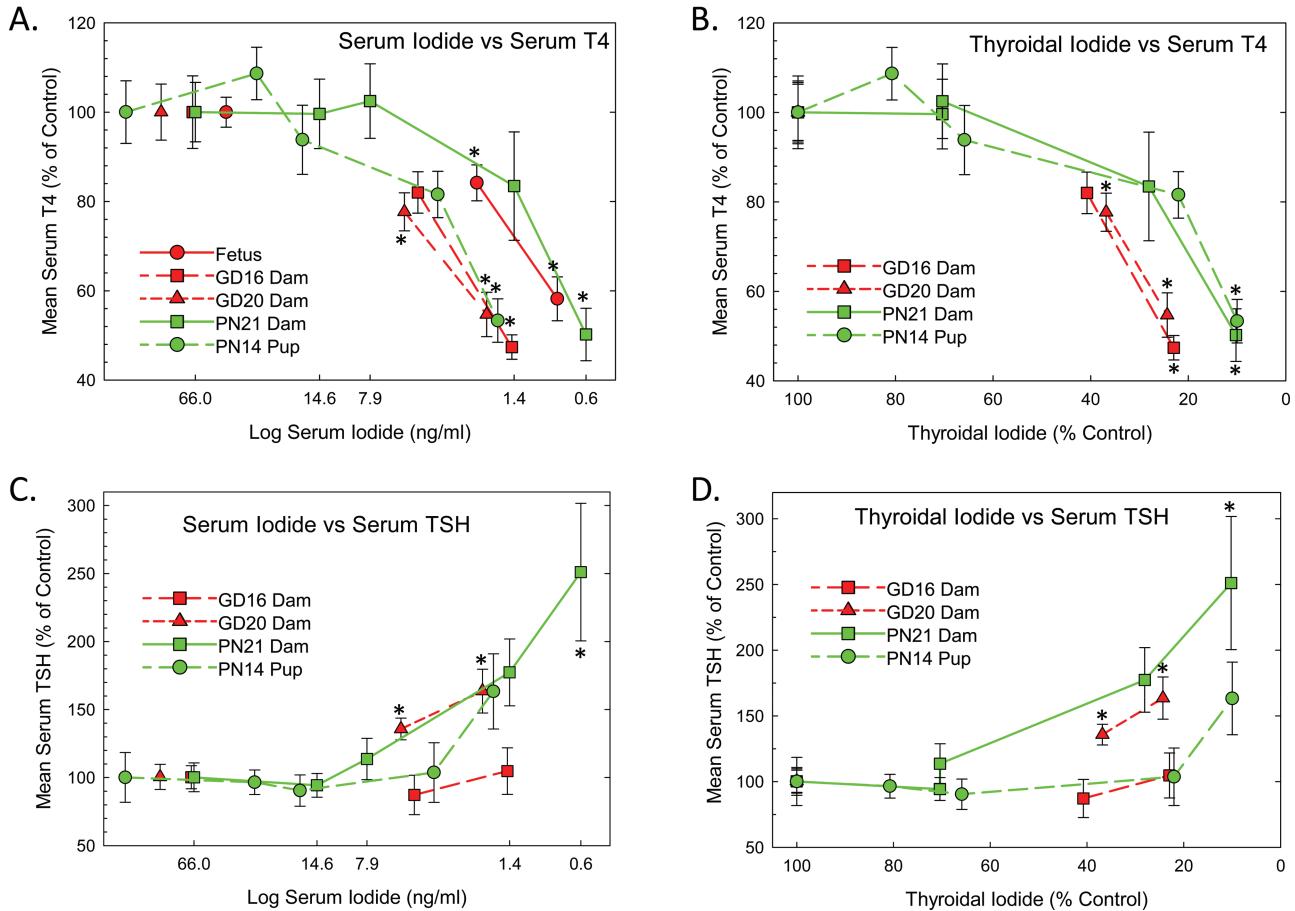


FIG. 8. Relating iodine in the thyroid gland to hormones in the serum. Mean (\pm SEM) serum T4 and TSH as a function of iodine in serum or thyroid gland. Asterisks represent significant differences from the control diet with highest level of dietary supplementation (D1) using Dunnett's mean contrast *post hoc* test following a significant main effect of diet in ANOVA. (A) Relationship of serum iodide and serum T4. Pregnant dams and neonates appear more sensitive to serum T4 declines under conditions of reduced serum iodide than do the lactating dam or the fetus. (B) In contrast, the pregnant dam shows declines in serum T4 with less severe drops in thyroid iodine stores than the postnatal dam or pup. TSH was more variable and not dramatically impacted by ID in any group. Lactating dams showed the largest increases in TSH (doubling) with reductions in serum (C) or thyroidal (D) iodine. A modest increase in TSH in late-gestation dams (GD20) was not seen at a slightly earlier stage of pregnancy (GD16).

most sparse but also the most essential for the development of quantitative dose-response models useful for extrapolation to humans.

Serum Indices of HPT Axis

Serum measures of iodide revealed dose-dependent reductions during pregnancy and lactation in dams, as well as in the late-term fetus and neonate. The largest and most reliable changes were seen as reductions in serum T4 in the two most ID diets. In the adult female, pregnant and lactating dam, and the neonate, serum and thyroidal T3/T4 ratios were increased with decreasing dietary iodine, indicative of induction of TSH-independent autoregulatory mechanisms in the thyroid gland. Consistent with hormone reductions in gland and

serum, the precursors for TH synthesis, MIT and DIT, were reduced in the thyroid gland of pregnant dams; DIT was more affected than MIT and paralleled the increase in T3/T4 ratios in gland and serum. A leftward shift in the curve describing the relationship between serum iodide and serum T4 suggests that pups and pregnant dams are more sensitive to ID than the lactating dam. A 40% reduction in thyroid iodine content was necessary to reduce serum T4 by 20% in the dam during gestation, whereas larger reductions in thyroidal iodine were required to disrupt serum T4 in the lactating dam and nursing pup.

In this study, we were not able to assess metrics of hormone regulation in the fetal thyroid gland. Schröder-van der Elst *et al.* (2001) reported that inorganic iodide in plasma

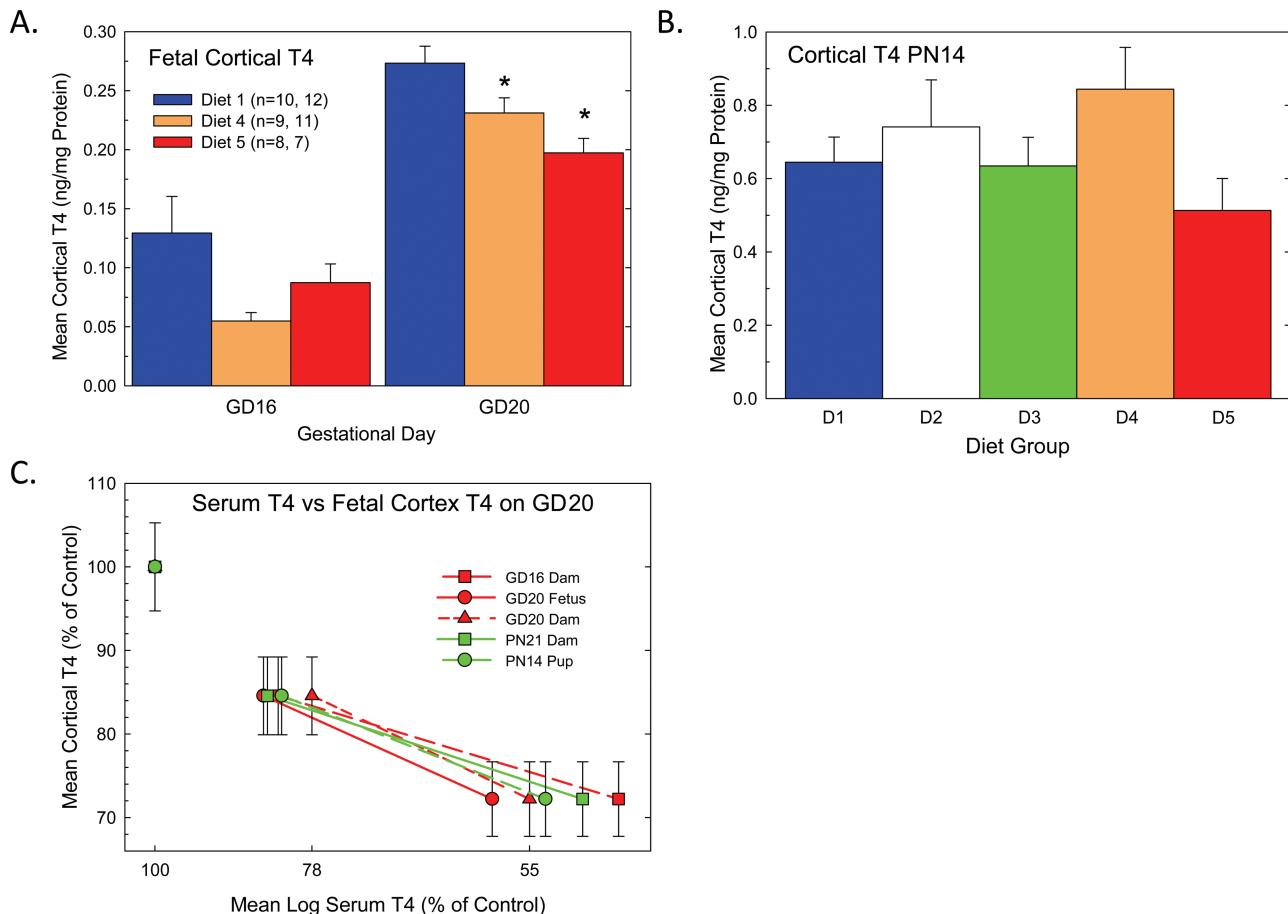


FIG. 9. THs in the brain. Mean (\pm SEM) T4 in cortex from fetal brain before (GD16) or after (GD20) the onset of fetal thyroid function (A) and in the cortex of the neonate on PN14 (B). T4 levels were higher in neonates than in the fetus and higher in late-stage than earlier-stage fetus. ID dose dependently reduced cortical T4 in the older fetus at both levels of ID tested ($F(2,27) = 7.06, p < 0.0034$). T4 in the brain of the younger fetus was also reduced but fell just short of statistical significance ($F(2,24) = 3.05, p > 0.06$), most likely due to high variability in the controls. In contrast, neonatal cortical T4 was not altered in the offspring of ID-treated dams on PN14 ($F(4,24) = 1.44, p > 0.25$). (C) Decline in fetal cortex T4 as a function of serum T4 shows that modest reductions in serum T4 are associated with significant reductions in cortical T4.

and absolute iodide uptake in the fetal thyroid gland are reduced at moderate levels of ID equivalent to our D5 (based on degree of maternal and fetal serum TH reductions). An upregulation of NIS in the placenta and fetal thyroid is not sufficient to normalize iodine uptake into the fetal gland. A higher induction of NIS and a larger thyroid gland in the mother results in no change in absolute thyroidal iodide uptake in the dam, but the fetal thyroid suffers more under conditions of competition with the dam for a limited iodine source. At more severe ID conditions, increases in expression of mRNA for deiodinase-I and thyroglobulin (not observed with modest ID) were seen, but increases were not sufficient to normalize thyroid iodine uptake (Schröder-van der Elst *et al.*, 2001). These data suggest that although the adaptive mechanisms to defend against ID are indeed present in the fetus (Escobar del Rey *et al.*, 1986; Obregon *et al.*, 2005; Schröder-van der Elst *et al.*, 2001; Versloot *et al.*, 1997), they may not be optimally triggered under

modest ID conditions or sufficiently mature to completely protect the developing fetus.

Brain Indices

The primary means by which TH influences brain development is by T3 binding to nuclear receptors to alter expression of thyroid-responsive genes (Williams, 2008). In adults, hypothyroidism is associated with large reductions in brain T4 concentrations before declines in brain T3 are evident (Broedel *et al.*, 2003; Pedraza *et al.*, 2006). Constant levels of the “active” hormone T3 are maintained in the face of falling T4 by upregulation of transporter proteins and the conversion of T4 to T3 by local deiodinases (Williams and Bassett, 2011). Because of these compensatory mechanisms, the small reductions in brain T4 we observed would not be expected to threaten the maintenance of an adequate supply of T3 to the neuron of the adult rat. However, this may not be the case in the brain of the fetus or neonate (see below).

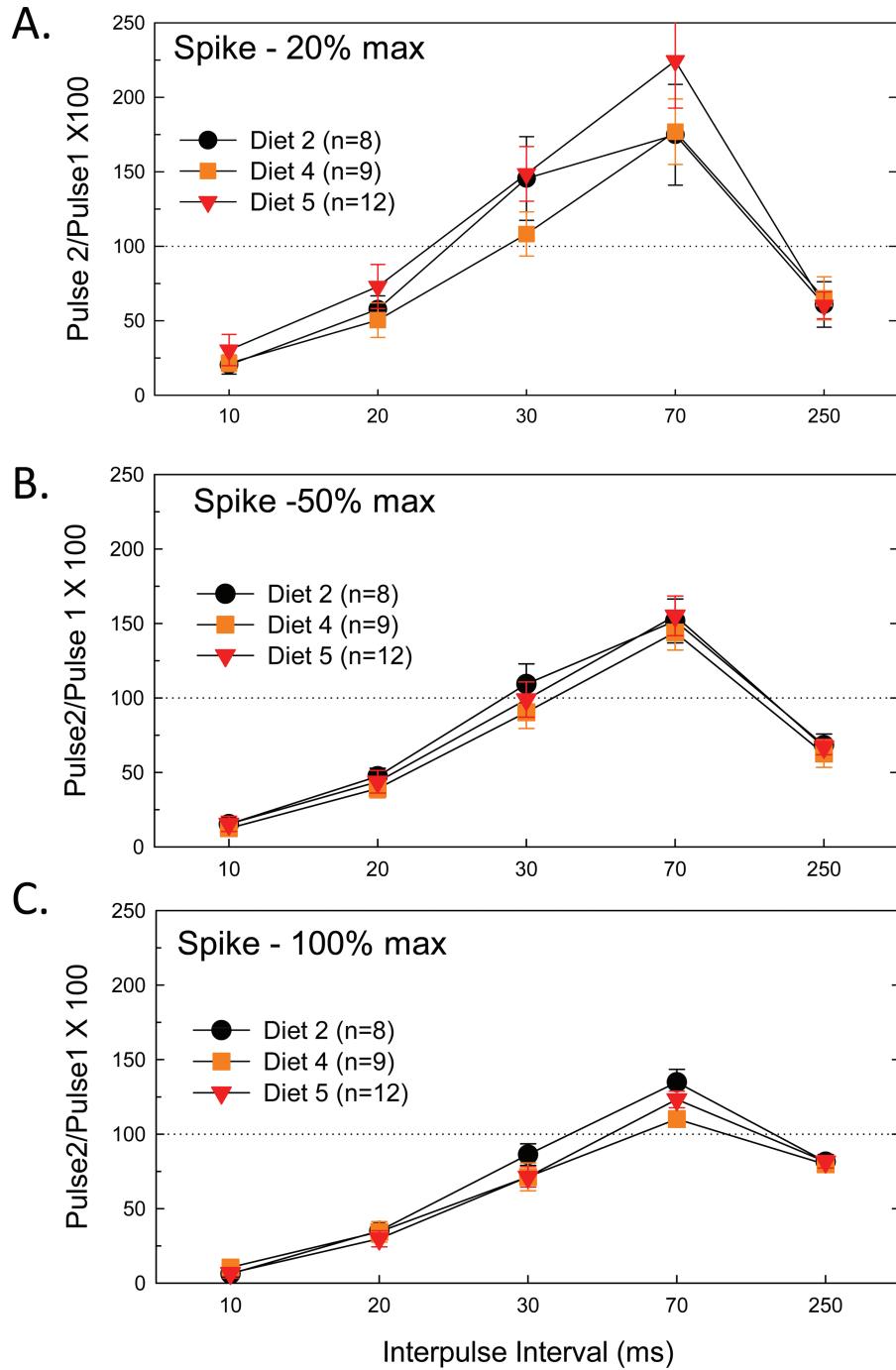


FIG. 10. Inhibitory synaptic transmission in adult offspring. Delivering pairs of stimulus pulses at varying intervals provides an index of inhibitory synaptic function and short-term plasticity inherent in the dentate gyrus. The magnitude of the second response of the pair is modulated by IPI and stimulus strength (pulse intensity producing [A] 20%, [B] 50%, or [C] 100% of maximal population response). A triphasic function is typically observed in the dentate gyrus with short intervals leading to suppression of the response, intermediate intervals to an augmentation, and even longer intervals to a second phase of modest suppression. This interval (IPI $F[4,104] = 150$, $p < 0.0001$) and stimulus-dependent (Intensity $F[2,52] = 17.32$, $p < 0.0001$) pattern were observed in this study, demonstrating clear electrophysiological control over the response. Paired pulse functions were not altered in iodine-deficient animal at any IPI or intensity (all $p > 0.32$ for diet, diet \times IPI interactions).

Neonatal brain TH. In a developmental model of severe ID, Escobar del Rey *et al.* (1987) reported that significant reductions in plasma and brain T4 were accompanied by relatively

minor reductions of T3 in the brain of the pup. Consistent with this report, we found significant reductions in serum T4 with no change in serum T3 did alter cortical levels of T4 in the neonatal

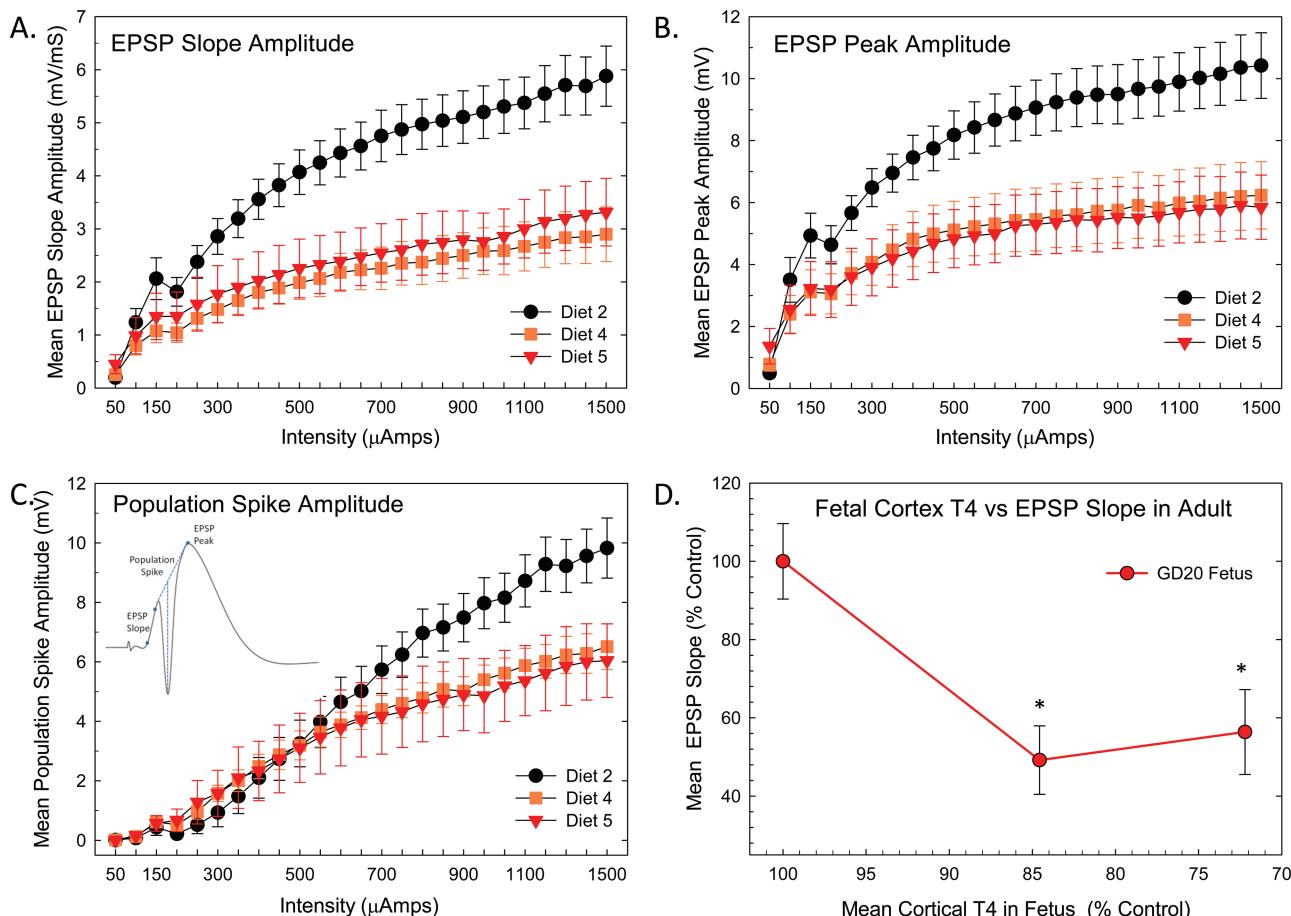


FIG. 11. Excitatory synaptic transmission in adult offspring. Compound field potentials evoked in the dentate gyrus from single-pulse stimulation (see inset C) of increasing intensity were recorded in adult offspring of ID-treated dams. Large amplitude potentials and orderly increases in three components of the field potential reflecting excitatory synaptic responsiveness (EPSP slope, A, and EPSP peak, B) and cell excitability (PS, C) demonstrate clear experimental control over the electrophysiological response. Although iodine-deficient diets were terminated at weaning and animals assessed in adulthood after full recovery of circulating THs, impairments in synaptic transmission were revealed by lower mean (\pm SEM) amplitudes in the input-output (I/O) curves. All three measures were significantly reduced, to equivalent degrees, in both iodine-deficient diets tested relative to controls. (D) Magnitude of decrease of the EPSP slope as a function of brain T4 measured in the fetal cortex. As little as \sim 15% reduction in cortical T4 is sufficient to permanently impair excitatory synaptic transmission in the adult offspring.

brain. Based on the findings of Escobar del Rey *et al.* (1987), the absence of change in cortical T₄ in our pups on PN14 suggests that brain T₃ in these brains (on PN14 at least) was also likely intact. Upregulation of the NIS in the mammary gland under ID conditions may serve to enrich the iodine supply in the milk of the lactating dam and provide a degree of protection to the developing pup brain (Dohán *et al.*, 2003; Escobar del Rey *et al.*, 1987; Schröder-van der Elst *et al.*, 2001).

Fetal brain TH. In contrast to findings in the adult (Pedraza *et al.*, 2006) and neonatal brain (Escobar del Rey *et al.*, 1986; present findings), significant reductions in cortical T₄ were observed in the fetal brain under the conditions of marginal ID described in this study. In a model of severe ID (i.e., complete suppression of serum T₄ and significant drops in serum T₃ in the dam), fetal brain T₄ and T₃ were reduced by 60–70% (Escobar del Rey *et al.*, 1986). This magnitude of change in

serum TH, although sufficient, may not be necessary to compromise optimal neurodevelopmental outcome. Our data suggest that decreases in serum T₄ as little as 20% in the pregnant dam or the fetus are sufficient to reduce fetal cortical T₄. In the adult or neonate, this magnitude of change in cortical T₄ would not be expected to alter brain T₃, but the same cannot be assumed for the fetus. Although we were not able to assess cortical T₃ in this study, the minor reductions in T₄ that we did observe in the fetal brain were associated with impairments in synaptic transmission in adult offspring (see below). Thus, brain development was indeed compromised under conditions of marginal ID that induced only mild serum and cortical T₄ reductions. Importantly, the dissociation between serum and cortical T₄ in the fetal versus neonatal brain also indicates that the standard serum indices of thyroid status routinely assessed during the postnatal period in the dam and neonate do not universally serve as accurate surrogates of hormonal status within the fetal brain.

Neurophysiological Impairments Accompany Minor Hormonal Insufficiencies

Electrophysiological indices of synaptic function were impaired in adult offspring from the two most ID conditions. At the time of testing, control levels of serum T4 had been re-established such that these deficits represent irreversible damage from developmental TH insufficiency. These changes observed in excitatory synaptic transmission are consistent with those reported in previous studies from our laboratory in which hypothyroxenemia in dams or pups was induced by propylthiouracil (Gilbert, 2011; Gilbert and Sui, 2006) or perchlorate (Gilbert and Sui, 2008). We did not see alterations in LTP, a model of synaptic plasticity in response to ID in the present study. Deficits in hippocampal plasticity in the dentate gyrus (Gilbert, 2011; Gilbert and Sui, 2006) and in a different hippocampal region (CA1) using an *in vitro* slice preparation have been reported following transient maternal hypothyroidism (Liu *et al.*, 2010; Opazo *et al.*, 2008) or with developmental hypothyroidism (Dong *et al.*, 2005; Sui and Gilbert, 2003; Sui *et al.*, 2005; Taylor *et al.*, 2008). Timing and degree of TH insufficiency, differential ontogeny of subregions in the hippocampus, and regional differences in responsiveness to TH (Iniguez *et al.*, 1996; Sharlin *et al.*, 2010) may all contribute to the differential pattern of effects across studies of hippocampal physiology.

Neurobehavioral Measures Remain Intact

In contrast to brain function assessed by electrophysiological means, no impairment in behavioral tests of sensory function or learning and memory was observed. Prepulse inhibition of the acoustic startle was enhanced, as expected, with increasing prepulse intensities and was comparable across all diet groups. In the Morris water maze, a standard test of spatial learning, clear indices of learning were evident in control and iodine-deficient animals, and no differences in acquisition or retention were evident. We and others have previously observed deficits in acoustic startle and spatial learning tasks in animals exposed developmentally to the goitrogens PTU, methimazole (MMI), or ID, but only with significantly greater degrees of hypothyroidism than induced here (Akaike *et al.*, 1991; Axelstad *et al.*, 2008; Dong *et al.*, 2011; Gilbert and Sui, 2006; Goldey *et al.*, 1995; Liu *et al.*, 2010; van Wijk *et al.*, 2008). We have also recently reported deficits in trace fear conditioning in adult offspring of PTU-treated dams but also at greater degrees of serum PN T4 reduction than achieved in this study (Gilbert, 2011). Collectively these data fail to provide any evidence of behavioral impairment as a function of this degree of TH insufficiency associated with the marginal ID conditions characterized in this model. They stand in contrast to electrophysiological indices of a persistent impairment in synaptic transmission in the hippocampus. One interpretation is that the degree of impairment in synaptic function observed here is of insufficient magnitude to have had any effect on brain development of any substantial consequence. Another interpretation is that the behavioral

tests examined here tests are not sensitive enough to detect subtle alterations in brain function. We submit that a persistent impairment in an integrated measure of communication of a large population of neurons within a critical synaptic circuit, demonstrated *in vivo*, long after return of normal thyroid status, cannot be readily dismissed as nonadverse just because it is not accompanied by deficiencies in simple tests of learning and memory. A similar degree of TH disruption in pregnant dams induced by other means has been associated with synaptic deficits, structural impairments, and altered gene expression in the brains of offspring (see Ausó *et al.*, 2004; Bastian *et al.*, 2012; Gilbert, 2011; Goodman and Gilbert, 2007; Opazo *et al.*, 2008; Royland *et al.*, 2008; Sharlin *et al.*, 2008). Neurodevelopmental consequences in children born to women with subclinical hypothyroidism or suffering from modest degrees of ID have also been difficult to document. Effects are subtle and the form they take is very much dictated by the timing of the hormone insufficiency (Zoeller and Rovet, 2004). It is also possible that the behavioral tasks evaluated in this study may be more conducive to detecting cognitive impairments mediated by postnatal hormonal insufficiencies in brain and deficits in synaptic plasticity, measures that were not altered in this study.

SUMMARY AND CONCLUSIONS

A model of marginal developmental ID is described. Various metrics of TH disruption in serum, thyroid gland, and brain in the dam, fetus, and neonate were evaluated. Importantly, a significant impairment in excitatory synaptic transmission was seen in adult offspring of iodine-deficient dams who displayed relatively modest degrees of TH reduction. A number of parameters were collected for application to a quantitative BBDR model of the developing HPT axis described in the accompanying article (Fisher *et al.*, 2012). Fetal serum T4 was the most sensitive peripheral marker of thyroid disruption. The fetus appears more at risk than the neonate under conditions of ID—brain T4 was not altered by ID in the neonate despite reductions in serum T4, but both serum and brain T4 were dose dependently reduced in the fetus. Reductions in fetal brain T4, although modest, were consistent with declines in fetal serum T4, and importantly, were associated with persistent deficits in synaptic function in the hippocampus. Our data suggest that a reduction in cortical T4 as little as 15% during fetal brain development is sufficient to induce permanent alterations in excitatory synaptic function in the adult offspring, and that this degree of fetal cortical T4 decline was present with serum hormone reductions of < 20% in the dam or fetus. These findings have implications for regulation of chemicals that perturb the thyroid axis and the identification of sensitive life stages (pregnant woman and her fetus) and populations (ID) who may be at greater risk to health consequences of xenobiotic exposure. They further suggest that standard behavioral tests of neurotoxicity appear relatively insensitive in their ability to detect brain impairment that accompanies a modest degree of developmental TH disruption.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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